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(54) Title: COMPOSITION AND METHOD FOR ALTERING DNA SEQUENCES BY HOMOLOGOUS RECOMBINATION

(57) Abstract

This invention relates to a composition and method for altering the sequence of a DNA fragment by homologous recombination. More particularly, this invention relates to a method for correcting genetic defects in mammals, particularly in man, by homologous recombination. This invention also relates to a gene therapy method for treating human genetic diseases, including cystic fibrosis, and other diseases, by homologous recombination.

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COMPOSITION AND METHOD FOR ALTERING DNA SEQUENCES BY HOMOLOGOUS RECOMBINATION

BACKGROUND OF THE INVENTION

This invention was developed at least partially with Government support under Grant Nos. DK39619 and HL42368 from the National Institutes of Health. The United States Government may have rights in this invention.

Field of The Invention

This invention relates to a composition and method for altering the sequence of a DNA fragment by homologous recombination. More particularly, this invention relates to a method for correcting genetic defects in mammals, particularly in man, by homologous recombination. This invention further relates to a gene therapy method for treating human genetic diseases, including cystic fibrosis, and other diseases, by homologous recombination.

Description of the Background

The alteration of defective genes associated with specific diseases has long been envisioned as a means for curing human genetic diseases. The knowledge and techniques of molecular biology have been brought to bear for several decades in the hope of correcting specific DNA defects that cause, or are associated with, human diseases. To date only complementation with exogenous DNA has been effective in altering a disease phenotype produced by a genetic defect.

Numerous defects in DNA sequences have been found in genes associated with human diseases such as thallassemias, sickle-cell anemia, cystic fibrosis, xeroderma pigmentosum, Fanconi's anemia, ataxia telangiectasia, and muscular distrophy. These deffects are inherited and persist through the generations.

Cystic fibrosis (CF), for instance, is an inherited disease afflicting about 1 in 2500 caucasian live births. CF is characterized primarily by defective regulation of cAMP dependent chloride ion transport, most notably in

epithelial cells. CF results in a debilitating loss of respiratory and pancreatic function, the primary cause of death being an opportunistic Pseudomonas aeruginosa infection of the airways. The disease is associated with mutations in the cystic fibrosis transmembrane conductive regulator (CFTR) gene, and the individuals afflicted with CF have a life expectancy of about 28 years.

The CFTR gene spans approximately 250 Kb and encodes a 6.5 Kb messanger RNA (mRNA) with an open reading frame of 4.6 Kb. At present, 27 exons that span the open reading frame (ORF) and the 3' untranslated region have been identified. The CF gene product is a protein of 170 Kd molecular weight with 1480 amino acids, that has two hydrophobic transmembrane domains and two nucleotide binding folds (NBF) containing ATP-binding consensus sequences. The two halves of the CFTR appear to have a quasi-structural symmetry although the actual sequence similarity between the two halves is modest, at best. Each half of the protein contains one transmembrane domain and one nucleotide binding fold (NBF). The two halves are linked by a large polar (R) domain. CFTR has structural homology with a number of other membrane associated proteins. The CFTR has been found to have an apparent structural similarity to the mammalian drug resistance P glycoprotein.

A mutation in exon 10, resulting in an in-frame deletion of phenylalanine at amino acid position 508 (ΔF508), is found in about 70% of all CF chromosomes. Of the now greater than 100 mutations found in the CFTR most appear to be associated with the NBF domains. ATP has been shown to be necessary for the PKA-dependent phosphorylation of CFTR and also plays an important role in the regulation of CFTR through the hydrolysis of ATP .

Genetic complementation and/or gene replacement/ conversion has been applied to alter the expression of endogenous mutant genes in cell lines and for the development of, e.g., transgenic mice. The use of a full-length wild type CFTR cDNA permits genetic

complementation to occur and to correct the defective phenotype corresponding to mutations on CFTR (Rosenfeld, M.A., et al., Cell 68:143-155(1992)).

The CFTR cDNA, in particular, has been transfected into cystic fibrosis cells to attempt complementation. The transfection of this cDNA, however, did not actually correct the defective CFTR gene sequence. Additionally, the introduction of a hybrid vector carrying the CFTR cDNA to attain complementation did not ensure the effective regulation of the CFTR gene.

Thus, while the CF phenotype was corrected with wild type CFTR cDNA by complementation, the problems of random integration and disruption of non-homologous genes still exist. In addition, the high levels of constitutive expression of CFTR in cells complemented with wild-type CFTR cDNA does not permit the regulation of the levels of gene expression and cellular function. These deficiencies can best be addressed by the specific correction of defective endogenous CFTR DNA sequences by homologous recombination as described herein.

The frequency with which homologous recombination occurs has been a major factor limiting the effectiveness of homologous recombination for gene therapy. The efficiency of homologous recombination is dependent upon a number of factors including the method of gene transfer or transfection, the cell in which it takes place, and the type of vector employed, if a vector is employed. Various methods have been successfully applied to the transfection of epithelial cells with exogenous DNA fragments. These include the utilization of calcium/strontium/phosphate, electroporation, protoplast fusion, liposome fusion, and microinjection. The methods showing the highest frequency of in vitro transfection in mammalian cells are microinjection and electroporation. Electroporation, however, is the more practical of the later two, since it is less labor intensive and affects a greater number of cells than microinjection. The method of choice for a particular cell type, such as epithelial cells, depend on

the biological characteristics of the cell, the frequency of homologous recombination and the ease of implementation of the various techniques.

In the past, attempts to perform sequence-specific repair of genomic DNA had focussed on the binding of oligonucleotides to desired sites in the DNA, and then site specific cleavage of the genomic DNA. Only homopyrimidine or guanine-rich oligonucleotides have been used successfully with this technique.

Up to the present time, however, homologous recombination has been generally limited in its application. To the inactivation or disruption of genes, generally known as gene "knockout" (Capecchi (1989); Boggs (1990); Thomas, Nature 336:847-850 (1990); Koller, PNAS 86:8932-8935 (1989); Mansour, Nature 336:348-352 (1988); Accili, PNAS 88:4702-4712 (1991)), and has been applied to mouse embryonic stem cells for the development of transgenic animals. Homologous recombination replacement and insertion vectors have been used in knockout experiments for the correction of mutant genes in rodents (Thompson, Cell 56:313-321 (1990); Koller, PNAS 86:8927-8931 (1989); Adair PNAS 86:4574-4587 (1989)). However, this approach has not been successfully employed for gene correction in metabolically active human cells given the disruption produced of intron sequences with selection markers. Only in vitro homologous recombination between two plasmids containing non-complementing, non-reverting deletions in an antibiotic resistance gene was reported with extract from human cells (Kucherlapati, Mol. Cell Biol. 5:714-720, (1985)). Single strand DNA (ssDNA) oligonucleotide fragments was also utilized to correct a mutation in an exogenous plasmid DNA co-transfected into human cells (Campbell, New Biologist 1:223-227 (1989)). While this study corrected a single 14 nucleotide insertion mutation and its phenotype with a 40 base single strand oligonucleotide, it does not demonstrate that this exchange can occur in chromatin DNA. As described above, naked ssDNA fragments have been cotransfected into human cells

with a plasmid carrying a mutant neomycin resistnace (neo^r) gene (Campbell, New Biologist 1:223 (1989)). The oligonucleotide fragments constituted only one strand of duplex DNA that was homologous to the mutated region of the neo^r gene. Co-transfection with the plasmid and the ssDNA fragments (40 nucleotides) was able to confer neo^r functionality onto a portion of the cells. However, neo^r can also be achieved by a mechanism that does not require homologous recombination (Lin. Mol. Cell. Biol. 4:1020-1034 (1986)). Thus, the two processes need to interact for homologous recombination to occur. In addition, the ssDNA fragments were not shown to cause a change in the cell phenotype when genes are integrated into or are a component of genomic DNA.

Single-stranded DNA coated with rec A was shown to anneal to homologous DNA and form a presumed triplex structure known as a "D-loop" (Moser, Science 238:6450659 (1987)).

The homologous oairing of genomic DNA sequences with a homologous rec A coated ssDNA fragment was inferred from the protection afforded the genomic HeLa cell DNA by the restriction enzyme Eco RI (Ferrini, Science 254:1494 (1991)). This D-loop structure, however, is unstable in vitro and may not have a long half-life in metabolically active cells.

The mammalian enzyme that has been implicated as having recombinase function also has exonuclease activiti (West, Ann. Rev. Biochem. 61: 603-640 (1992) and may impose a different conformational structure on chromatin DNA than does rec A.

The ability of recombinases to pair any single-stranded oligonucleotide with a homologous duplex DNA to form a three-stranded complex has been applied to the specific targeting of any desired DNA sequence. Recombinases such as the Rec A protein can facilitate annealing of a single strand of DNA to an intact duplex to form a novel D-loop structure in which the third strand includes both purines and pyrimidines (Hsieh, P., and Camerini-Otero, R.D., J.

Biol. Chem. 264:5089 (1989); Hsieh, P., et al., Genes Dev.4:1951 (1990); Rao, B.J., et al., Proc. Natl. Acad. Sci. 88:2984 (1991)).

Ferrini and Camerini-Otero described a method to target any Eco RI site for specific cleavage (Ferrini and Camerini-Otero, Science 254:1494 (1991)). The technique of Ferrini and Camerini-Otero is based on the ability of a Rec A protein from E. coli to pair any single-stranded oligonucleotide in vitro to a homologous duplex DNA to form a D-loop structure.

Accordingly, there is still a need for an in situ method of correcting genetic defects by homologous recombination in mammalian cells, particularly in human cells. When applied in vivo, the present method is useful for gene therapy to treat human genetic diseases and for countering the deleterious effects of these diseases. In addition, when applied in vitro the method is suitable for ex vivo gene therapy as well as for producing transgenic animals.

SUMMARY OF THE INVENTION

This invention relates to a composition for altering a DNA sequence flanked by first DNA sequences upstream and downstream thereto, comprising

a substantially pure, isolated DNA fragment comprising an altering DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the altering DNA sequence, the altering DNA sequence lacking complete homology to a DNA sequence to be altered, and each second flanking DNA sequence being subtantially homologous to the corresponding first flanking DNA sequence, and comprising one terminus of the isolated DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular metabolic conditions; and

a biologically acceptable carrier.

This invention also relates to a method for genetically altering a double-stranded DNA sequence in a mammalian

cell, comprising

obtaining a mammalian cell comprising a double-stranded DNA fragment that comprises a DNA sequence to be altered and first flanking DNA sequences upstream and downstream thereto,

obtaining a second DNA fragment comprising an altering DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the altering DNA sequence, the altering DNA sequence lacking complete homology to the DNA sequence to be altered, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comprising one terminus of the second DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular metabolic conditions;

delivering the second DNA fragment into the cell under conditions effective for the second DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking DNA sequences; and

allowing the corresponding flanking DNA sequences to anneal under cellular conditions and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically altered cell comprising the altering DNA sequence in place of the DNA sequence to be altered.

Also provided herein is an altered mammalian cell line obtained by the method described above. The altered cell line has a biologically functional gene substituted for a defective one.

This invention also encompasses a mammalian cell line provided with a genetic defect associated with a predetermined disease, the gene being substituted for the wild type by the method described above.

This invention also relates to an ex vivo method for genetically altering a DNA sequence in a subject's target cells, comprising

obtaining non-transformed or immortalized cells from

a mammalian subject, the cells comprising a DNA fragment that comprises a DNA sequence to be altered and first flanking DNA sequences upstream and downstream from the DNA sequence to be altered;

obtaining a second DNA fragment and comprising an altering DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the altering DNA sequence, the altering DNA sequence lacking complete homology to the DNA sequence to be altered, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comprising one terminus of the second DNA fragment, and at least a number of nucleotides effective attain annealing to the corresponding first flanking DNA sequence under cellular metabolic conditions;

delivering the second DNA fragment into the cell under conditions effective for the second DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking DNA sequences;

allowing the corresponding flanking DNA sequences to anneal under cellular conditions and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically altered cell comprising the altering DNA sequence in place of the DNA sequence to be altered, and

returning the genetically altered non-transformed or immortalized cells to the subject.

This invention also encompasses an ex vivo method for gene therapy of a disease associated with a defective DNA sequence of a DNA fragment present in a mammalian subject's target cells, the fragment comprising the defective DNA sequence and first flanking DNA sequences upstream and downstream from the defective DNA sequence, the method comprising

obtaining target cells from a mammalian subject in need of the therapy, the cells comprising a DNA fragment that comprises a defective DNA sequence associated with the

disease and first flanking DNA sequences upstream and downstream from the defective DNA sequence;

obtaining a second DNA fragment comprising a functional DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream thereto, the functional DNA sequence lacking complete homology to the defective DNA sequence, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comprising one terminus of the second DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular conditions;

delivering the second DNA fragment into the cell under conditions effective for the DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking DNA sequences;

allowing the corresponding flanking DNA sequences to anneal and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically functional cell comprising the functional DNA sequence of the second species; and

returning to the subject the gene therapy treated stem cells lacking the defective DNA sequence substantially ameliorating the disease.

This invention also provides a method of producing a transgenic, non-human, mammalian animal, comprising

obtaining a germ cell or embryo cell of a non-human, mammalian animal of a first species, the cell comprising a DNA fragment comprising a DNA sequence to be altered and first flanking DNA sequences upstream and downstream thereto;

obtaining a second DNA fragment, comprising an altering DNA sequence from a mammalian of a second species, two termini, and second flanking DNA sequences located upstream and downstream thereto, the altering DNA sequence lacking complete homology to the DNA sequence to be altered, and

each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comprising one terminus of the second DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under metabolic cellular conditions;

delivering the second DNA fragment into the cell under conditions effective for the DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking DNA sequences;

allowing the corresponding flanking DNA sequences to anneal and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically altered cell comprising the altering DNA sequence of the second species;

replacing the genetically altered germ or embryo cells in a non-human, mammalian animal; and

allowing for gestation to proceed to term to thereby produce a non-human, mammalian, transgenic animal of the first and second species.

This invention also encompasses a transgenic animal obtained as described above.

Still part of this invention is an in vivo method for altering a DNA fragment present in a subject's target cells, the DNA fragment comprising a DNA sequence to be altered and first flanking DNA sequences upstream and downstream from the DNA sequence to be altered, the method comprising

obtaining a second DNA fragment comprising an altering DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the altering DNA sequence, the altering DNA sequence lacking complete homology to the DNA sequence to be altered, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comprising one termini of the second DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular

conditions;

administering to the subject a composition comprising the second DNA fragment under conditions effective for the second DNA fragment to reach the subject's target cells and allowing the DNA fragment to enter the subject's cells and the second flanking sequences to locate the first flanking DNA sequences; and

allowing the corresponding flanking DNA sequences to anneal under cellular conditions, and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce genetically altered target cells comprising the altering DNA sequence.

This invention relates as well to a method for gene therapy of a disease associated with a defective DNA fragment in a subject's target cells, the DNA fragment comprising a defective DNA sequence and first flanking DNA sequences upstream and downstream from the defective DNA sequence, the method comprising

obtaining a second DNA fragment comprising a functional DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the altered DNA sequence, the functional DNA sequence lacking complete homology to the defective DNA sequence, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comrpising one terminus of the second DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular conditions;

administering to the subject the second DNA fragment under conditions effective for the second DNA fragment to reach the subject's target cells, and allowing the DNA fragment to enter the subject's cells and the second flanking sequences to locate the first flanking DNA sequences; and

allowing the corresponding flanking DNA sequences to anneal, and the second DNA fragment and the cellular DNA to

undergo homologous recombination under cellular conditions to produce biologicallly functional target cells comprising the functional DNA sequence, and substantially ameliorate the disease's symptoms.

Still part of this invention is an immortalized human cell line carrying two alleles of the corresponding defective cellular cystic fibrosis DNA and identified as CFTE29o-, and having an ATCC Accession No. CRL 11151.

Other objects, advantages and features of the present invention will become apparent to those skilled in the art from the following discussion.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention arose from a desire of the inventors to provide a composition and method for substituting one DNA sequence for another in existence in a mammalian cell.

In one embodiment, the present invention is intended for correcting defects associated with genetic human diseases, such as thallasemias, sickle cell anemia, cystic fibrosis, Fanconi's anemia, cystic fibrosis, retinitis pigmentosa, xeroderma pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, Duchenne's muscular dystrophy, Tay-Sach's disease, and the like. The inventors envisioned the application of the present method to the therapeutic treatment of inherited diseases, that are extremely debilitating or produce the patient's premature death, some of these diseases lack a cure at the present time.

The method of the present invention can alter DNA sequences utilizing small DNA fragments comprising, e.g., functional or quasi-functional DNA sequences such as wild type sequences for each particular DNA fragment associated with a disease. In this embodiment, the method of the invention substitutes functional DNA sequences for disfunctional ones by homologous recombination. Once in place, the new DNA sequences are, typically, effective for restoring normal or quasi normal function in mammalian cells, such as human cells. In this embodiment, the method

of this invention in situ corrects genetic defects by allowing a subject's own cells to replace a defective gene or fragment thereof with a functional DNA segment, e.g., when the cellular DNA replicates and the cells reproduce themselves.

The present method may be practiced in vitro, ex vivo or in vivo, depending on the application, e.g., ease of reaching the desired target cells for homologous recombination.

The present approach implements homologous recombination as an alternative to gene complementation with cDNA, a superior alternative at that, because it produces a complete replacement of a defective DNA sequence. Moreover, the present method places an exogenous functional DNA sequence under the regulatory control of the endogenous gene promoter and ensures that the gene is expressed at appropriate levels in the cells. None of these effects are accomplished by complementation techniques utilized up to the present time.

In another embodiment, the present method may be applied to immortalized mammalian cell lines to manufacture cell lines carrying a defect associated with a specific disease. The thus constructed cell line may be utilized for testing drugs intended for use against such disease, or for other purposes.

In another embodiment, the present technology may be applied to the ex vivo production of transgenic animals as further described below.

The cells may be transfected with the DNA fragment comprising the altering DNA sequence and flanking DNA sequences by methods such as electroporation, (Iannuzzi, M.C., et al., Annual Rev. Respir. Dis. 138:965-8(1988)), microinjection, (Folger, K. et al., Molec. and Cell Biol. 2:1372(1982), by complexing the DNA to be inserted with $Sr^{++}/Ca^{++}/PO_4$ (Brash, D. E., et al., Mol. Cell Biol. 7:2031-4(1987); Reddel, R.R., et al., Cancer Res. 48:1904-9 (1988)), the DNA is complexed with protein and then coated with a lipid (Legendre, PNAS (1992) in press); or

encapsulating the DNA with a lipid layer (Segmer et al., PNAS (USA) 84: 7413 (1987)), or, e.g., in the case of human cells by using hybrid vectors carrying or altering DNA fragment (Sambrook et al., in Molec. Cloning, Cold Spring Harbor Laboratories (1989)).

One type of hybrid vectors for human a humanized cells carrying a functional DNA sequence are replacement vectors. The hybrid vectors are constructed in a manner such that the DNA sequence to be replaced and any sequences from the vector other than the altering DNA sequence are eliminated during DNA replication and homologous recombination. Another type of hybrid vectors are insertional vectors that also carry the functional DNA sequence but which rely on intrachromosomal recombination to eliminate selection markers and to duplicate genomic sequences.

The technology for conducting every step of the present method is either provided herein or is publically available. Thus, within the text of the present description, or by example below, the inventors teach how to prepare the DNA fragment comprising an altering DNA sequence for specific gene replacement, how to conduct the substitution of the altering DNA sequence, how to increase the frequency of homologous recombination, how to detect the occurrence of homologous recombination and gene replacement, and the like.

In one embodiment, in order to target specific gene sequences for replacement, at least parts of the DNA segment surrounding the DNA sequence to be replaced must be known. A minimum number of base pairs must be matched to the flanking DNA sequences upstream and downstream from the portion of the DNA sequence to be replaced. In this particular embodiment, a portion of the endogenous flanking DNA sequence regions must be elucidated in order to synthesize the altering DNA fragment, and in particular the DNA flanking sequences attached thereto.

Alternatively, when no portion around the DNA fragment targeted for replacement can, or is desired to, be ascertained, a wild type or functional allele DNA fragment

may be utilized on the assumption that a large portion of the functional DNA fragment will be homologous to that of the defective DNA fragment, and therefore act as flanking DNA sequences.

Similarly, when a defective cell line or transgenic animal is desired to be created, one or more defective alleles may be utilized as a replacement DNA fragment.

Also disclosed herein is the creation of a novel immortalized human cell line, ΣCFTE29o-, that originated from a cell from a CF human patient with two ΔF508 alleles, that was transformed to become immortalized. This cell line is thus homozygous for the ΔF508 deletion that is the most common genetic defect found in cystic fibrosis patients. This cell line has been deposited with the ATCC in Rockville, MD, and has been given an Accession No. CRL 11151.

The establishment of the above immortalized cell line carrying a genetic defect physiologically resembling the human error permitted the present inventors to generate the technology necessary to correct genetic defects associated with different diseases.

The correction of a genetic defect by the method of the invention is exemplified herein for the cystic fibrosis (CF) disease by substitution with a normal 491 base pair DNA fragment of CFTR spanning the defective DNA sequence of the genomic DNA of a CF cell line, i.e., exon 10 (193bp) and the 5'(163bp) and 3' (135 bp) flanking intron. This substitution is attained by homologous recombination. This immortal cell line is a cystic fibrosis (CF) line derived from a tracheal ephitelial homozygous for the ΔF508 mutation. This is a post crisis cell line that is defective in cAMP-dependent Cl^- ion transport, secretes chloride in response to a calcium ionophore, and expresses CFTRmRNA. The cells were prepared from CF tracheal ephitelial cells from an autopsy specimen and were isolated uncultured by a known procedure (Gruenert, et al., In Vitro Cell Dev. Biol. 26:411 (1990); Yamaya, et al., Am. J. Physiol. Lung Cell Mol. Physiol. 261:L485 (1991)). A pure

culture of ephitelial cells may be transfected by calcium phosphate precipitation with a linearized plasmid such as pSBori- as previously described (Cozens, et al., PNAS (USA) 89:5171 (1992); Gruenert, et al., In Vitro Cell Dev. Biol. (1990), supra). The transfected cultures may be grown in an appropriate medium at, e.g., 37oC until cells with the desired growth characteristics appear (Gruenert, D.C. et al., PNAS (USA) 85:9591 (1988); Gruenert, D. C., BioTechniques 5:740 (1987)). The transformants may be isolated, e.g., by trypsinization and regrown as is known in the art.

Methods to detect a genetic alteration or correction produced by homologous recombination in accordance with this invention are also disclosed herein. DNA or RNA segments isolated from transfected cells may be amplified by the polymerase chain reaction (PCR), a method known in the art (e.g., US Pat. No.4,965,188). Appropriate allele-specific oligonucleotide primers for conducting the PCR permit the synthesis of recombinant and unaltered cellular DNA fragments, that are prepared as is also known in the art. These fragments may be separated and identified, e.g., by gel electrophoresis, and the presence of fragments may be confirmed by hybridization with oligonucleotide probes specific for recombinant or altered sequences. The thus produced recombinant cells may additionally be tested for the requisite phenotypic defects, e.g., altered chlorine transport associated with the CF gene defect.

This invention also provides a composition for altering a DNA sequence flanked by first DNA sequences upstream and downstream thereto, comprising

a substantially pure, isolated DNA fragment comprising an altering DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the altering DNA sequence, the altering DNA sequence lacking complete homology to a DNA sequence to be altered, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comprising one terminus of the isolated DNA fragment and at

least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequences under cellular metabolic conditions; and

a biologically acceptable carrier.

A broad range of altering DNA fragment sizes is envisioned herein. For instance, fragments of up to about 1,000 base pairs and longer, and as small as about 1 to 300 base pairs are contemplated. However longer or shorter fragment lengths are also suitable as long as annealing and homologous recombination occur.

Biologically acceptable carriers are known in the art and an artisan will be able to select those suitable for the present purpose. Typically, the carriers are inert compounds or compositions that do not interact with the DNA fragment and do not interfere with its circulation in the subject's blood or its penetration of the subject's cells. Suitable carriers are liquid, solid or aerosols.

When in liquid form the composition may further comprise salts and other agents which maintain pH and osmolarity, and the like. Preferred carriers are aqueous solutions such as saline, phosphate buffer, and others. However, others may also be used as long as they have a pH that is compatible with the cell growth medium or in the in vivo case, the subject's blood. Typically, the pH is about 6 to 8 and more preferrably about 7.

In addition, other agents may also be incorporated into the therapeutic composition, such as a calcium and/or strontium/ and phosphate agent in proportions to the DNA fragment known in the art.

In another embodiment of the invention, the DNA fragment to be altered comprises an exon having one terminus at each end, wherein the exon comprises the DNA sequence to be altered and each of the first flanking DNA sequences is provided with one inner terminus that is located at least about 5 base pairs, and more preferably about 10 base pairs, still more preferably up to about 17, and in some instances 25 and more, base pairs outside the corresponding exon's terminus.

This is intended for minimizing errors in the base pairing of the daughter strains produced when homologous recombination ocurrs close to the end of the coding DNA fragments.

This embodiment of the invention places the flanking DNA sequences outside regions, e.g., exons. Thus, cushion sequences are provided, which place the site of initial enzymatic events in homologous recombination in sequences of simple homology that are not involved in any modification, e.g., non-transcribed sequences or introns. Should mismatching occur during the early chain elongation steps, these mutated sequences will be placed in the introns, thus diminishing the likelihood of inducing new mutations in the altered gene. Those skilled in the art would know that where the sequence to be altered is itself found in the intron sequence, the flanking DNA sequences would be selected such that nearby exon sequences are functioning as flanking DNA sequences or the flanking DNA sequences will be entirely outside the DNA to be altered.

DNA flanking sequences may be utilized having at least about 5 base pairs long, preferably at least about 10 base pairs long, and still more preferably at least about 50 base pairs long.

When at all possible, flanking DNA sequences of up to about 100 base pairs and even larger may also be utilized to attain better pairing and annealing between the flanking DNA sequences of the endogeneous DNA and the DNA fragment inserted in the cell.

The altering DNA fragment of the composition of the invention may be a double-stranded DNA fragment, a single-stranded DNA fragment or complementary single-stranded DNA fragments. The selection of an altering DNA may depend on the source of the DNA, the method of amplification of the DNA and/or the experimental/clinical aim of the user of the method. Most preferably, double-stranded DNA and complementary single-stranded DNA is used.

Further included in the invention is a composition comprising the altering DNA fragment and a recombinase

enzyme. Examples of recombinase enzyme are UVSX, Rec A, yeast recombinase, human recombinase and RAD 51 yeast recombinases including DMC and HPP1, although other enzymes are also suitable. The recombinase is preferably present in a ratio of about 1 protein molecule to every 3 bases. Other ratios, however, may also be used. When the DNA fragment is double-stranded the DNA is, most likely denatured prior to contacting it with the recombinase. The foregoing recombinases have been shown by the prior art to promote pairing of single-stranded DNA to double-stranded homologous DNA but not of two double-stranded oligonucleotides. These proteins are thus useful to increase the frequency of pairing between the altering DNA fragment and the cellular DNA fragment to be altered. The enhanced pairing by recombinases is especially useful where the frequency of recombination is low. The use of recombinases may also be beneficial to boost the frequency of recombination where biologically effective therapy requires the alteration of a high percentage of target cells.

In another embodiment of the present composition, the altering DNA fragment is enveloped by a lipid layer, encapsulated by a lipid and a protein layer, or is compounded as a DNA fragment-$Ca_3(PO_4)_2$ and/or -$Sr_3(PO_4)_2$ complex. The choice of the foregoing preparations will vary depending on the cell type used, the in vitro or in vivo conditions and the inherent limitations of each transfection method.

Preferred conditions for enveloping the second DNA fragment with a lipid layer are as follows. The second DNA fragment is admixed with a lipid such as dioleophosphatidyl ethanolamine, dipalmitoylphosphatidylethanolamine (dipalmitoyl PtdEtn), palmitoyloleoylphosphatidylethanolamine (palmitoyloleoyl PtdEtn), dioleoylphosphatidylcholine (PtdCho), dimyristoylphospatidylethanolamine (dimyristoyl PtdEtn), diphytanoylgycero-phosphatidylethanolamine (diphytanoyl PtdEtn), N-monomethyl PtdEtn, and N-dimethyl PtdEtn in a proportion of about 1 μg: 1nmole to 1μg: 500 nmoles, in an aqueous solution. Other components and

proportions are discussed below when this technology is applied to the in vivo method. The pH of the solution may be adjusted to about 8 to 10, and more preferably about 9. In addition to the above, ingredients such as a buffer and other known components may also be added to this composition. The amounts in which these components may be added are standard in the art and need not be further described herein.

The second DNA fragment may be, coated with a recombinase or uncoated, complexed with a protein, such as gramicidin S, and then coated with a lipid such as described above, in a proportion of about 1 μg : 0.5μg : 1 nmol to 1μg : 50 μg : 500 nmol, and more preferably about 1μg : 1μg :10 nmol to 1μg : 20μg : 250 nmol. The combination of the DNA with the lipid and protein components may be done in one step or by first contacting theDNA, whether coated with a recombinase or uncoated, with the protein, and adding thereafter the lipid.

In another preferred embodiment of the invention, the DNA sequence to be altered of the above composition comprises a DNA sequence encoding the cystic fibrosis transmembrane (CFTR) conductance regulator protein and the altering DNA sequence comprises a functional (normal) allele of the CFTR gene. Thus, a wild type allele may be utilized herein.

The above composition may also be compounded to alter simultaneously more than one cellular DNA sequence. A majority of CF mutations have been sequenced and located to exon 10. Using available DNA libraries and the normal CFTR gene sequence functional wild-type DNA fragments homologous to CF mutations can be prepared.

This invention also provides to a method for genetically altering a double stranded DNA sequence in a mammalian cell, comprising obtaining a mammalian cell comprising a double-stranded DNA fragment that comprises a DNA sequence to be altered and first flanking DNA sequences upstream and downstream thereto;

obtaining a second DNA fragment comprising an altering DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the altering DNA

sequence, the altering DNA sequence lacking complete homology to the DNA sequence to be altered, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comprising one terminus of the DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular conditions;

delivering the second DNA fragment into the cell under conditions effective for the second DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking sequences; and

allowing the corresponding flanking DNA sequences to pair and anneal and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically altered cell comprising the altering DNA sequence.

This method may be practiced by contacting about 1 to 200 μgDNA/10^6cells, and more preferably about 5 to 50 μg DNA/10^6cells. The above DNA amounts and cells may be contacted in an aqueous growth medium at a concentration of about 0.1 μg/ml to 100 μg/ml of the DNA, and preferably about 5 to 20 μg/ml of DNA. The proportion may vary depending on the cell type to be transfected, and the transfection delivery/ administration method used.

When the method is practiced by microinjecting the DNA, about 10 to 100,000 DNA fragments, and preferably about 1,000 to 10,000 DNA fragments, may be microinjected into each cell nucleus. The DNA may be delivered in a volume of about 0.1 to 100 femtoliters and more preferably of about 1 to 10 femtoliters per nucleus.

During transfection the cells may be grown in an appropriate cell culture medium such as is shown in the examples at about 32 to 42°C. and more preferably about 37°C. However, other media may also be used where desirable or where components used for transfection so require. The incubation temperature may also vary according to the requirements of the transfection method used but may generally be in the range of about 32 to 40°C. The cells may be incubated with

the altering DNA fragment for a period of about 1 to 10 days preferably about 2 to 7 days. Culture conditions which maximize cell growth rate, generally optimize homologous recombination.

When a recombinase is utilized, care should be exercised to maintain it at about 4°C or lower, and then bring it to reaction temperature when ready for use.

The mammalian cell whose DNA sequence is to be altered may be obtained from immortalized or non-transformed cells of a specific mammalian strain or species, including single species mammalian cells including human cells, and humanized mouse or rat cell lines, among others. The latter may by utilized for the in vitro experimental manipulation of cells to produce variant or hybrid cells having a genetic defect associated with a certain disease. In addition, if the cells are embryonic or germ cells, they may be utilized for the production of transgenic animals. In addition, the cells may be extracted from an animal or human, such as by a spinal tap, a venous or arterial injection, from a specialized tissue, and the like. When precursor cells are utilized, the modifications incorporated into these cells will eventually produce cells of a certain specificity. This is the case of many blood cells, where stem cells are the ones being modified.

The second DNA fragment may be obtained from a cell, by PCR amplification as is known in the art. When manufacturing an altered cell line having a defect associated with a disease, the altering DNA fragment may be obtained from a cell from a patient afflicted with the disease in question and inserted by homologous recombination in a normal cell by applying the method of the invention. Similarly, when a cell defect is desired to be corrected, a wild-type animal cell, including human cells, may be isolated, its DNA excised with restriction enzymes and utilized in the method of this invention. Alternatively, the entire altering DNA fragment, the altering DNA sequence and/or the second flanking DNA sequences may be synthesized to have a specific organization. The altering DNA fragment may then be

amplified, e.g., by the PCR technique as is known in the art.

The delivery of the altering DNA fragment into the cell may be conducted by a variety of techniques discussed above. These encompass providing the second DNA fragment enveloped by a lipid layer, complexed with a protein and encapsulated by a lipid or as a DNA fragment/strontium/calcium/phosphate complex. The altering DNA fragment may also be provided as a hybrid vector, where the vector is capable of replication and expression in a mammalian cell and has operatively linked thereto the altering DNA fragment. The conditions for contacting any of these compositions with the cells to be altered were described above and need not be repeated herein.

Whether or not homologous recombination has occured may be checked as described above and in the examples section below by testing a specific function of the cell which has been modified, and/or by analyzing the cellular DNA and/or RNA of the altered cell, or by enzyme restriction of the cellular DNA or a gene product expressed by the cell, enzyme restriction and electrophoresis of the protein material and comparison to molecular weight markers to identify the origin of the protein in question.

The foregoing method may also be used to alter DNA sequences in non-nuclear cellular DNA, present in the cell as mitochondrial DNA or as a virus, plasmid or a hybrid vector.

It will be apparent to a person skilled in the art that this method also provides a means for altering DNA sequences which do not express a gene product, including alterations in regulatory sequences, intron sequences, and the substitution of redundant codon sequences. Other possible corrections include the insertion of functional DNA sequences other than normal wild-type DNA, which permit an at least quasi-normal function and have an at least beneficial therapeutic effect on the subject. Generally, the altering DNA sequence need only be of at least sufficient size and of a sequence such that, after homologous recombination, it will provide a hybrid cell having a genetic DNA capable of functional gene expression.

The above method may be practiced to insert an altering DNA sequence of at least about 1000 base pairs/nucleotides, more preferably DNA sequences of at least about 500 base pairs, and even smaller DNA sequences of about 100 to 300 base pairs. Even a 1 base pair DNA sequence may be substituted by the method of the invention. More preferably still, DNA sequences of 1 to 99 base pairs may be used herein. Moreover, the DNA to be inserted may have a deletion, in which case the altered cellular DNA will actually be smaller than the original cellular DNA or it may have an addition in which case the altered cellular DNA will be longer than the original cellular DNA. No change in cellular DNA length occurs when the defect is a simple mutation.

As already indicated, the flanking DNA sequences comprise at least about 5 or more base pairs, preferably about 10 or more base pairs, and more preferably 17 or more base pairs in length, and even up to about 100 and more. The length of flanking sequences may be varied depending on factors such as the length of the altering sequence, the location of the cellular DNA fragments to be altered, and the particular base sequence occurring within the flanking DNA sequences as is known in the art.

It is known that random recombination may occur when flanking sequences anneal to other similar DNA sequences. A person skilled in the art will know that increasingly longer flanking sequences increasingly promote annealing to short random homologous DNA regions. To minimize random pairing, somewhat longer flanking sequences of at least 8 to 17 base pairs, and longer, are preferred.

The synthesis of the altering DNA fragment may be conducted by methods known in the art, such as the isolation and separation of a wild-type DNA fragment by cleavage with restriction endonucleases, site-directed mutagenesis, PCR amplification, de novo oligonucleotide synthesis, and/or combinations of enzyme restriction and ligation to produce deletions, additions or the like.

In a preferred embodiment of the foregoing method, the DNA fragment to be altered comprises an exon having two

termini, wherein the exon comprises the DNA sequence to be altered, and each first flanking DNA sequence is provided with one inner terminus that is located at least about 5 base pairs, and more preferably about 10 base pairs, and still more preferably about 50 base pairs, outside the exon's termini. In this embodiment of the invention, the flanking sequences are linked outside the transcribed sequences (exons) or those DNA sequences singled out for alteration, thus providing an oligonucleotide cushion, thereby placing the site of initial enzymatic events of replication and/or homologous recombinaton in non-transcribed sequences (introns) or on sequences that are not singled out for alteration. Should mismatching occur during the early chain elongation steps, these mutated sequences will be placed in introns, diminishing the likelyhood of introducing new mutations in the altered gene. Those skilled in the art would know that where the sequence to be altered is itself located in intron sequences, the flanking DNA sequences will be placed such that nearby exon sequences are either entirely between flanking sequences or entirely outside both the DNA to be altered and its flanking sequences.

The altering DNA fragment may be a double-stranded DNA fragment, a single-stranded DNA fragment, or single-stranded complementary fragments. The selection of DNA fragment may depend on the source of the DNA, the method of amplification of the DNA and/or the experimental/clinical application of the method. Where the second DNA fragment is double-stranded, the method may further comprise denaturing the second DNA fragment prior to delivering it to the cell. The denaturation may be conducted, as is known in the art, by heating and quenching, or by other methods.

Also a part of this invention is the foregoing method, wherein the altering DNA fragment is contacted with a recombinase enzyme prior to delivery into the cell. The recombinases may be selected from the group consisting of UVXS, Rec A, yeast recombinase, human recombinase, and RAD^{51} yeast recombinases including DMC and HPPI, among others, as described above. In a preferred embodiment, the altering

DNA fragment is a single-stranded DNA fragment that is contacted with a recombinase enzyme. However, the recombinase may be combined with double-stranded DNA after separation of the strands under conditions known in the art. The foregoing recombinase agents are useful to increase the frequency of pairing between the intended altering DNA sequence and the cellular DNA. This enhancement of pairing may be useful where the frequency of recombination is found to be low. The use of recombinases may also be beneficial to boost the frequency of recombination when the biological application of the method requires relatively high recombination frequencies.

In a preferred embodiment of the foregoing method, the altering DNA fragment is delivered into the cell complexed with calcium/ strontium/phosphate, or with protein and encapsulated by a lipid, enveloped by a lipid layer or by electroporation or microinjection.

When the altering DNA fragment is delivered into a cell by electroporation, the conditions may be as shown in the examples.

When the altering DNA fragment is delivered into the cell by microinjection, the conditions shown in the examples may be utilized. Other conditions may also be utilized as is known in the art.

The choice of the transfection method will depend on the type of cell to be transfected, the frequency of recombination required and the number of cells to be transfected, as is known in the art. Nuclear microinjection will produce higher rates of transfection and thus requires a lesser DNA:cell ratio. As microinjection is a manipulative technique practiced with a relatively small number of cells, this technique is preferable to conserve scarce amounts of DNA. Other methods such as calcium/strontium/phosphate complexes, lipid envelopment, lipid-protein encapsulation and electroporation require higher DNA concentrations per set number of cells. These methods, while somewhat less efficient, are particularly applicable to large numbers of cells since the cells need not be individually manipulated.

The DNA-lipid and lipid-DNA-protein transfection techniques are especially applicable to in vivo transfection since less cell lethality is encountered, the DNA is protected from DNase degradation, and the method is compatible with intracorporeal injection or administration.

The technology for practicing the above steps is generally known in the art and need not be described here in detail.

In one aspect of the invention, the above method is practiced with an altering DNA sequence differing from the DNA sequence to be altered by at least 1 base pair, and the genetic alteration introduced by the altering DNA sequence is selected from the group consisting of a substitution, a deletion, and an addition.

The altering DNA sequence may comprise at least about 1000 base pairs to lengths approaching that of a gene. More preferably, the altering DNA sequence is about 100 to 300 base pairs long and longer, and more preferably yet the altering DNA sequence may be about 1 to 99 base pairs long, permitting the alteration of small DNA sequences within the cellular DNA. Moreover, the altering DNA sequence may, upon homologous recombination, result in a net decrease in cellular DNA size where the altering DNA sequence possesses a deletion or in an increase if it possesses an intercalation.

The synthesis of the altering DNA sequence may be undertaken by methods known in the art, such as the isolation of a wild type functional DNA segment by cleavage with restriction endonucleases as is known in the art, and site directed mutagenesis, PCR amplification, de novo oligonucleotide synthesis and/or a combination of enzyme restriction and ligation to produce deletions or additions, and the like.

In one embodiment of the above method, the DNA sequence to be altered is associated with a disease such as Fanconi's anemia, cystic fibrosis, retinitis pigmentosa, xeroderma pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, Duchenne's muscular distrophy, or Tay-Sach's disease, among others. The method may also be used to alter the DNA sequences of cells with other genetic defects. The

method only requires that a normal DNA sequence be known or that a normal wild-type DNA fragment be available from known DNA libraries. Other DNAs may be used in this method so as to produce an alteration in cellular DNA with an associated modification of the defective gene function.

In an additional embodiment of the above method, the mammalian cell comprises immortal and non-transformed cells, and amongst them germ line cells, embryo cells, and the like, or somatic cells such as stem cells and the like. Preferred cells are human cells, and amongst the human cells are non-transformed cells.

Genetic alterations in accordance with this invention may be attained with transformed cell lines and non-transformed cells, and amongst them with both germ line cells and somatic cells in vivo and in vitro.

This invention further includes a mammalian cell line obtained by the above method, wherein the altering DNA sequence contains a faulty sequence associated with one of the diseases listed above, e.g., cystic fibrosis. The cell may be made to contain two cystic fibrosis alleles and thus is homozygous for cystic fibrosis.

The production by the method of this invention of cell lines carrying defective genes associated with CF and other diseases is also contemplated herein. Thus, mammalian, e.g., human, cell lines may be obtained by the present method carrying genetic defects associated with specific diseases as described above. Particularly preferred cells are those having physiological and/or biochemical characteristics associated with CF, Fanconi's anemia, retinitis pigmentosa, xeroderma pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, Duchenne's muscular dystrophy, and Tay-Sachs disease. These alterations are introduced in the chromatin of the cell and are placed under the regulatory control of the corresponding gene effecting a correction of the genetic defect.

Also embodied in this invention is an ex vivo method for genetically altering a DNA sequence in a subject's target cells, comprising

obtaining cells from a mammalian subject, the cells comprising a DNA fragment that comprises a DNA sequence to be altered and first flanking DNA sequences upstream and downstream from the DNA sequence to be altered;

obtaining a second DNA fragment comprising an altering DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the altering DNA sequence, the altering DNA sequence lacking complete homology to the DNA sequence to be altered, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comprising one terminus of the second DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular conditions;

delivering the second DNA fragment into the cell under conditions effective for the second DNA fragment to enter thereto and for the second flanking DNA sequence to locate the first flanking sequences;

allowing the corresponding flanking DNA sequences to pair and anneal, and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically altered cell comprising the altering DNA sequence; and

returning the genetically altered cells to the subject.

The foregoing ex vivo method may be used to remove the subject's own cells, alter the DNA of those cells and subsequently return the altered cells to the subject. The method may also be used to alter related transformed cells from a subject or from established cell lines and then alter them to, e.g., suppress their malignant characteristics, and the like, and then place the altered cells in the subject. By performing homologous recombination outside of the subject's body, this method has the advantage of permitting different transfection methods and conditions that would not be feasible or easy to implement with an in vivo method. The synthesis of the altering DNA fragment may be accomplished by methods known in the art, such as the isolation and

separation of a wild-type DNA fragment by cleavage with restriction endonucleases, PCR amplification, site-directed mutagenesis, de novo oligonucleotide synthesis, and/or a combination of enzyme restriction and ligation to produce deletions, additions or the like. All these techniques are known in the art.

The various steps of this method may be practiced as described previously under a broad range of conditions.

The genetically altered non-transformed or immortalized cells may then returned to the subject by injection and/or surgery as is know in the art.

Also preferred is an ex vivo method for genetic therapy of a disease associated with a defective DNA sequence of a DNA fragment present in a mammalian subject's target cells, the fragment comprising the defective DNA sequence and first flanking sequences upstream and downstream from the defective sequence, the method comprising

obtaining target cells from a mammalian subject in need of therapy, the cells comprising a DNA fragment that comprises a defective DNA sequence associated with a disease and first flanking sequences upstream and downstream from the defective sequence;

obtaining a second DNA fragment comprising a functional DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the functional DNA sequence, the functional DNA sequence lacking complete homology to the defective DNA sequence, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comprising one terminus of the second DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking sequence under cellular conditions;

delivering the second DNA fragment into the cell under conditions effective for the second DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking DNA sequences;

allowing the corresponding flanking DNA sequences to anneal, and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically functional cell comprising the functional DNA sequence; and

returning to the subject the gene therapy treated cells lacking the defective DNA sequence and substantially ameliorating the disease's symptoms.

In one aspect, the method is applied to altering stem cells which have the potential to repopulate, such as hematopoeitic stem cells. However, other somatic cells may be altered by homolgous recombination if that particular cell type has the potential to repopulate in the subject. Other cell types may also be altered if upon homologous recombination, their accumulated cell function is sufficient to overcome the disease condition.

The method requires that the wild type sequence of the disease be known or that the wild type DNA fragment be available from known gene libraries to provide functional DNA sequences. This gene therapy method may be utilized to correct a wide variety of genetic diseases, somatic mutations, and viral sequences whether nuclear, mitochondrial, or cytoplasmic. Thus, also within the contemplated applications of this invention is the alteration of DNA sequences associated with diseases in animals, or of human genetic diseases originating from other animals, where there is lodging in the human body of at least a genetic piece originating from another source.

In order to practice the ex vivo method for gene therapy of a disease associated with a defective DNA sequence, the target cells may be obtained from a mammalian subject, including a human, afflicted with the disease. Cells may be obtained as discussed above, either from a bodily fluid, specific tissues carrying somatic cells, and the like. An artisan would know what specific cells are desired and how to extract them from a subject.

The functional or second DNA fragment may be utilized as a composition, and its synthesis or isolation are as described above.

The technology for delivering the functional or second DNA fragment into a cell was also discussed above, and encompasses the oral, systemic and dermal routes, among others. As already indicated above, the composition comprising the second DNA fragment may also comprise other ingredients such as recombinase enzymes, calcium/strontium/phosphate, a lipid or lipo-proteic agent for enveloping the DNA fragment, and the like.

Also embodied in this invention is a method for producing a transgenic non-human, mammalian animal, comprising

obtaining a germ cell or embryo cell of a non-human, mammalian animal of a first species other than human, the cell comprising a DNA fragment comprising a DNA sequence to be altered and first flanking DNA sequences upstream and downstream thereto;

obtaining a second DNA fragment comprising an altering DNA sequence from a mammalian animal of a second species, two termini, and second flanking DNA sequences located upstream and downstream from the altering DNA sequence, the altering DNA sequence lacking complete homology to a DNA sequence to be altered, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, one terminus of the second DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular conditions;

delivering the second DNA fragment into the cell under conditions effective for the second DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking DNA sequences;

allowing the corresponding flanking DNA sequences to pair and anneal and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically altered cell comprising the altering DNA sequence;and

replacing the genetically altered cell in the non-human, mammalian animal; and

allowing gestation to proceed to term to thereby produce a transgenic non-human mammalian animal of the first and second species, where the recombined DNA may be human.

The method for producing a transgenic animal provided herein permits the insertion of a desired gene or portions of a gene having a desired characteristic from other species or other strains of the same species. In one aspect of this method, different DNA fragments having desirable characteristics may simultaneously be incorporated into the cellular DNA by homologous recombination. The present method thus enables the insertion of multiple genes and, therefore, the transfer of novel biological or biochemical functions to a new species.

In another aspect of the method, the construction of a hybrid recombinant DNA fragment constructed so as to link flanking DNA sequences to the altering DNA sequences to be inserted is also contemplated. This is so since a DNA sequence from another species or strain is less likely to carry substantially homologous flanking DNA sequences which will anneal to the cellular flanking DNA sequences. The linking of endogenous flanking DNA sequences to a foreign altering DNA sequences may be performed by techniques known in the art, such as the isolation of DNA fragments by cleavage with restriction endonucleases, PCR amplification, de novo oligonucleotide synthesis, and/or a combination of enzyme restriction and ligation.

In a preferred aspect, the second DNA fragment is delivered into the cell complexed with $Ca^{++}/Sr^{++}/PO_4^{=}$, enveloped by a lipid layer, complexed with a protein and encapsulated by a lipid or by microinjection or electroporation.

In the case of ex vivo methods, microinjection is especially preferred as a transfection techniques for its high rate of homologous recombination. The small number of target cells required by this technology may overcome its labor intensive nature.

The isolation of embryo and germ cells as well as the different manipulations involved in the application of the transfection methods are known in the art and exemplified herein.

In another embodiment of this invention, it is provided herein an in vivo method of altering a DNA fragment in a subject's target cells, the DNA fragment comprising a DNA sequence to be altered and first flanking DNA sequences upstream and downstream from the DNA sequence to be altered, the method comprising

obtaining a second DNA fragment comprising an altering DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the altering DNA sequence, the altering DNA sequence lacking complete homology to the DNA sequence to be altered, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking sequence, and comprising one terminus of the second DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular conditions;

administering to the subject the second DNA fragment under conditions effective for the second DNA fragment to reach the subject's target cells and allowing the DNA fragment to enter the subject's cells and the second flanking DNA sequences to locate the first flanking DNA sequences; and

allowing the corresponding flanking sequences to anneal and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce genetically altered target cells comprising the altering DNA sequence.

The administration to the subject of the second DNA fragment is done by means of a composition comprising the DNA fragment and a pharmaceutically-acceptable carrier and/or other agents such as recombinase enzymes, calcium phospate and/or strontium phosphate, a lipid agent, a lipid and protein agent, and the like, that were described above.

Typically, the carrier may comprises solid, liquid or gaseous carriers. Examples of carriers are aqueous solutions, including water, buffered, aqueous solutions and the like.

While it is possible for the active ingredient to be administered alone it is preferable to present it as a pharmaceutical formulation. The formulations of the present invention comprise at least one active ingredient, as defined above, together with one or more acceptable carriers thereof and optionally other therapeutic agents.

Examples of other therapeutic agents suitable for use herein are any compatible drugs that are effective by the same or other mechanisms for the intended purpose, or drugs that complementary to those of the present agents.

Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Formulations include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and parenteral (including subcutanecus, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include bringing into association the active ingredient with the carrier which constitutes one or more accesory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liqiud carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, dragees or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water in-oil liquid emulsion. The active ingredient may also be presented as a bolus or paste. A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by

compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, e:g., providone, gelatin, hydroxypropylmethyl cellulose, lubricant, inert diluent, preservative, disintegrant, e.g., sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. The tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other that the stomach. This is particularly advantageous for purine nucleoside derivatives, since such compounds are susceptible to acid hydrolysis.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquied carrier. For inhalation, the formulation may be in the form of an aerosol that will reach the lungs.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending and/or thickening agents. The formulation may be presented in unit dose or multidose sealed containers,

for example, ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art for the type of formulation in question, for example, those suitable for oral administration may include such further agents as sweeteners, thickeners and flavoring agents.

In addition, the above compounds according to the invention and their pharmaceutically acceptable derivatives may be employed in combination with other therapeutic agents for the treatment of the indicated conditions. Examples of such further therapeutic agents include agents that are effective for the treatment of or associated conditions. However, other agents that contribute to the treatment of the disease and/or its symptoms, such as inhibitors of neutrophyl function, retinoic acid, anti-inflammatory agents, adenosine agonists, and the like, are suitable.

The compounds other than those of the invention suitable for such combination therapy may be administered simultaneously, in either separate or combined formulations, or at different times, e.g., sequentially, such that a combined effect is achieved.

The compound according to the invention, also referred to herein as the active ingredient, may be administered for therapy by any suitable route, including oral, rectal, nasal, respiratory, topical (including buccal and sublingual), vaginal and parenteral (including subcutanecus, intramuscular, intravenous and intradermal). It will be appreciated that the preferred route will vary with the condition and age of

the recipient, the nature of the infection and the chosen active ingredient including other therapeutic agents.

In general, a suitable dosis for each of the above-mentioned conditions will be in the range of about 5 μg to 1000 mg/kg body weight of the recipient, e.g., a human, per day, preferably about 10 μg to 500 mg/kg body weight/day, and still more preferably of about 50 μg to 200 mg/kg body weight/day. The desired doses may be administered as 1 to 6 or more subdosis administered at appropriate intervals through the day. Repeated administration of the composition of the invention may be undertaken at intervals of about a few days or weeks if a substantial degree of substitution is not detected when target cells are tested after the treatment.

The remaining steps in this method may be conducted as described above and exemplified hereinafter.

The foregoing method may also be used to alter DNA sequences in non-nuclear DNA whether present in the cell as mitochondrial DNA, viruses, plasmids, or hybrid vectors. In addition, this method also provides a means for altering DNA sequences which do not result in the expression of altered gene products. These sequences include defective regulatory genes, intron sequences and the substitution of redundant codon sequences, among others. Other alterations may be undertaken by practing the above method, such as the insertion of sequences other than normal wild type DNA but which permit quasi-normal cell function or any other desired cellular function.

In its application, the above method may be practiced by inserting an altering DNA sequence of at least about 1000 base pairs. More preferably, smaller DNA sequences of about 100 to 300 base pairs may be inserted, and still more preferably DNA sequences of about 1 to 99 base pairs may also be inserted. The alteration provided in the foreign DNA sequence may be a deletion, in which case the altered cellular DNA will actually be smaller than the original cellular DNA. Similarly, when the altering DNA sequence is longer than the

cellular one to be replaced, the altered cellular DNA will be shorter than the unaltered one.

The second flanking DNA sequences comprise at least about 5 or more base pairs, preferably about 10 or more base pairs, and more preferably 17 or more base pairs in length. Flanking DNA sequences of these lengths are adequate to acheive annealing. The length of the flanking DNA sequences may be varied depending on factors such as the length of the altering DNA sequence, the location of the cellular DNA fragments, and the particular base sequences within the altering and flanking DNA sequences. An artisan would know how to determine a minimun length herein.

As is known in the art, random recombination may occur when flanking sequences anneal to other similar sequences in cellular DNA. To minimize the random integration of foreign DNA, somewhat longer flanking sequences of at least 8 to 17 base pairs are preferred, and up to 50 or 100 base pairs may be used. A person skilled in the art will know that increasingly longer flanking sequences further minimize the potential risk of annealing of altering DNA fragments to short random homologous regions.

The altering DNA fragment may be synthesized by methods known in the art, such as the isolation of a wild-type DNA fragment by cleavage with restriction endonucleases, site-directed mutagenesis, de novo oligonucleotide synthesis, and/or a combination of enzyme restriction and ligation to produce deletions, additions, or the like.

The DNA fragment may be administered in accordance with the above method orally, topically, intratumoraly, intracavitarily, subcutaneously, intravenously, intramuscularly, intranasaly, or by respiratory inhalation, among other routes.

For oral administration, the altering DNA fragment may be compounded with an inert diluent or with an edible carrier, that may be enclosed in gelled capsules, or compressed into tablets or incorporated in the diet. Other excipients may be added to the formulation such as those utilized for ingestible tablets, troches, capsules, elixirs, suspensions,

syrups and wafers, among others. Also contained in the preparation may be a binder such as gum tragacanth, acacia, corn starch or gelatin, excipients such as dicalcium phosphate, disintegrating agents such as corn starch, potato starch, alginic acid and the like, lubricants such as magnesium sterate, sweetening agents such as sucrose, lactose or saccharin, flavoring agents such as peppermint, olive, wintergreen or cherry flavoring as well as other known artificial and natural flavoring compounds. A liquid carrier may also be added to the capsules. Sustained-release preparations and formulations are also within the confines of this invention.

Coatings or otherwise modified forms of the preparation are also contemplated herein such as coatings of shellac, sugar and the like.

A syrup or elixir may contain the altering DNA fragment, sucrose as a sweetening agent, methyl or propyl parabens as preservatives, a dye and a flavoring such as cherry or orange flavors, among others. Any material added to the pharmaceutical composition should be pharmaceutically-acceptable and substantially non-toxic in the amounts employed. Sustained-release preparations and formulations are also within the confines of this invention.

Injectable preparations of the altering DNA fragment may be prepared for administration to a variety of body site by intravenous, introthoracic subcutaneous, intramuscular, intraocular, or intraperitoneal injection. Such injectable preparations of the composition as a free acid or a pharmaceutically-acceptable salt may be administered in a pharmaceutically acceptable solvent with or without a surfactant such as hydroxypropyl cellulose.

Dispersions are also contemplated such as those utilizing glycerol, liquid polyethylene glycols and mixtures thereof and oils. Antimicrobial compounds may also be added to the preparations.

The injectable preparations may include sterile aqueous solutions or dispersions and powders which may be diluted or suspended in a sterile environment prior to use.

Carriers such as solvents or dispersion media containing, e.g., water, ethanol polyols, vegetable oils and the like, may also be added. Coatings such as lecithin and surfactants may be utilized to maintain the proper fluidity of the solution. Isotonic agents such as sugars or sodium chloride may also be added as well as products intended for the delay of absorption of the active compounds such as aluminum monostearate and gelatin. Sterile injectable solutions are prepared as is known in the art and filtered prior to storage and/or administration. Sterile powders may be vacuum-dried or freeze-dried from a solution or suspension containing them. Such solvents may also contain surfactants, dispersing agents, gelling agents, thickening agents and compounds suitable to maintain physiological osmolarity, pH and isotonicity. Such solution, cream or ointment may be applied to the skin, or to the surfaces of the eye.

Pharmaceutically-acceptable carriers as utilized in the context of this patent include any and all solvents, dispersion media, coatings, antimicrobial agents, isotonic and absorption delaying agents and the like as is known in the art. All preparations are prepared in dosage unit forms for uniform dosage and ease of administration. Each dosage unit form contains a predetermined quantity of active ingredient calculated to produce a desired therapeutic effect in association with a required amount of pharmaceutical carrier.

Suspensions of the altering DNA fragment may be prepared using pharmaceutically-acceptable/carriers, utilizing glycerol, liquid polyethylene glycols, oils and mixtures thereof with or without surfactants, wherein the carrier is capable of suspending powdered preparations of one or more components of the composition or suspending insoluble liquid dispersions (droplets) containing one or more components of the composition. Such suspensions may also contain surfactants, dispersing agents, gelling agents, thickening agents and compounds suitable to maintain physiological osmolarity, pH and isotonicity. Suspensions containing the composition or components thereof may thus be formulated into

a fluid.

The method also comprises the administration of the composition of the invention by an intranasal or inhalation route. For intranasal or inhalation administration the DNA agent is compounded with an inert pharmaceutically-acceptable diluent wherein the diluent forms a solution or suspension using as a diluent a solvent or suspension medium containing, e.g., water, ethanol, polyols, oils and other similar compounds capable of aerosol formation. Such aerosol solution or medium may contain compounds suitable to maintain physiological osmolarity, pH and isotonicity.

The foregoing method may be applied to a human afflicted with a genetic disease such as Fanconi's anemia, cystic fibrosis, retinitis pigmentosa, xeroderma pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, Duchenne's muscular dystrophy and Tay-Sachs' disease, among other genetic diseases.

In a more preferred embodiment, the method of the invention is applied to the alteration of the genetic defect associated with CF disease. The method may also be applied to cells with other genetic defects for which the wild-type or otherwise normal DNA sequence is known. Also within this invention is the alteration of DNA sequences associated with genetic diseases in animals other than humans.

The foregoing in vivo method may be practiced where the DNA fragment to be altered comprises an exon having two termini, and the exon comprises the DNA sequence to be altered, and each first flanking DNA sequence is provided with one inner terminus that is located at least about 5, and preferably at least about 10 base pairs or more outside the corresponding exon's terminus. In this embodiment of the invention, the flanking DNA sequences are placed outside the transcribable sequences (exons), thus providing a DNA sequence cushion, which places the site of the initial enzymatic events of replication in non-transcribable regions (introns). Should mismatching occur during early chain elongation steps, these mutated sequences would occur in the introns, thus the likelyhood of inducing new mutations in

the altered gene are reduced. Those skilled in the art would know that where the sequence to be altered is itself located in an intron sequence, the flanking DNA sequences will be defined such that the nearby exon sequence is either entirely between flanking sequences or entirely outside both the DNA to be altered and its flanking DNA sequences.

Also embodied in the method of this invention is the use of a second DNA fragment which may be a double-stranded DNA fragment, a single-stranded DNA fragment, and a single-stranded complementary DNA fragment having a certain degree of homology but not complete homology, to the DNA sequence to be altered and substantially complete homology to the thus defined flanking DNA sequences. The selection of the above DNA fragments will depend on the source of the DNA, the method of transfection of the DNA, and/or the experimental/clinical aim to which the method is being applied.

The in vivo method may be practiced where the altering DNA sequence differs from the DNA sequence to be altered by at least one base pair, and the genetic alteration introduced by the altering DNA sequence may be a substitution, a deletion, or an addition. The altering DNA fragment may be synthesized by methods known in the art, such as the isolation and separation of a wild-type DNA fragment by cleavage with restriction endonucleases, site-directed mutagenesis, de novo oligonucleotide synthesis, and/or a combination of enzyme restriction and ligation to produce deletions, additions or the like.

Other DNA sequences may also be utilized lacking the "defect" of the DNA sequence to be altered. These may still provide normal or quasi-normal cell function or any other desired function and, upon homologous recombination, serve as a functional DNA sequence.

The second DNA fragment may be administered as a composition such as was described above. By means of example, the composition may comprise the second DNA fragment as a complex with $Ca^{++}/Sr^{++}/PO_4^{=}$, or where the second DNA fragment is enveloped by a lipid layer or encapsulated by a lipid and protein layer. The selection of the composition to be

administered will depend on factors such as the type of cell to be transfected, the frequency of transfection and recombination required and the number of cells to be transfected.

For the practice of the above in vivo method for altering a cellular DNA sequence, the administration of the altering DNA fragment enveloped by a lipid layer or encapsulated in a lipid and protein layer are preferred.

The encapsulation of the DNA with lipid and lipid-protein is especially applicable to in vivo transfection since less cell lethatity is encountered, the DNA is protected from DNase degradation and the method is compatible with intracorporeal injection or administration.

One concern about the direct delivery of genes in vivo is the ability of the polynucleotide to survive in circulation long enough to arrive at the desired cellular destination.

In the following paragraphs, different manners of aiding the delivery of DNA fragments to the inside of the cell are discussed and exemplified by means of publications. The contents of the publications relevant to the practice of this invention, including components, conditions and manners of developing each technique are incorporated herein by reference.

In this respect, the coating or masking of the polynucleotide is of extreme utility. The utilization of liposomes or a lipoproteic coating is extremely useful. In addition, a successful liposome system uses the cationic lipid reagent dioleyloxytrimethalammonium (DOTMA). DOTMA may be mixed with phosphatidyl ethanolamine (PE) to form the reagent Lipofectin^{R}. When this reagent is utilized to carry the polynucleotides the liposomes are simply mixed with the DNA and readied for administration. No encapsulation of the DNA inside the liposome is required. This reagent has been used to transfect reporter genes into human lung epithelial cells in culture, to introduce the CAT gene into rats by the intratracheal route, and to introduce the CAT gene into mice intratrachealy and intraveinously. Cytotoxicicity, however, may be a problem with high loads of Lipofectin^{R}.

In another embodiment, the composition comprising the polynucleotide may also contain lipopolyamine, lipophylic polylysines and a cationic cholesterol, all of which have been applied to gene transfer and culture.

In addition to the above, efficient gene transfer requires the targeting of an altering DNA fragment to the cell of choice. This can be attained by procedures based upon receptor mediated endocytosis (Wu et al., J. Biol. Chem., 262:4429(1987); Wu et al., J.Biol.Chem., 263:14,621 (1988)). This technology utilizes a cell-specific ligand-polylysine complex bound to the polynucleotide through charge interactions. This complex is taken up by the target cells as reported by Wu et al. in the case of a human hepatoma cell line and rat hepatocytes in vivo using asialoorosomucoid as a ligand. The successful transfection of a similar hepatoma cell line resulting in stable expression of enzymatic activity was reported following insulin-directed targeting (Huckett et al, Biochem. Pharmacol.40:253(1985)). Wagner et al., PNAS (USA) 87:3410 (1990) and Wagner et al., PNAS (USA) 88:4255 (1991) utilized a transferrin-polycation to attain the delivery of a plasmid into a human leukemic cell line and observed expression of the encoded luciferase gene. These proteins require attachment to the polynucleotide via, e.g., a polylysine linker.

Moreover, in many receptor-mediated systems as chloroquine or other disrupters of intracellular trafficking may be required for high levels of transfection. Adenovirus, for instance, has been used to enhance the delivery of polynucleotides in receptor-mediated systems (Curiel, et al., PNAS (USA) 88:8850 (1991)).

The directed delivery of polynucleotides into a cell is aided by the neutralization of the large negative charge present on the polynucleotide. Similarly, the ability of the polynucleotide to permeabilize the membrane of the targeted cell increases the delivery of the polynucleotide into the cell. Polycations have been used to neutralized the polynucleotide charge and aid in the membrane permeabilization and translocation. (Felgener, PNAS (USA)

84:7413 (1987)) cationic lipid has also been used for this purpose (US Patent No. 4,946,787; Behr, J.P., Tets, Lett. 27:5861(1986)).

Ligands are known to aid in direct on of the polynucleotide into a desired targeted area within the cells. Typically, to direct the polynucleotide into the genomic DNA of the cell, liegands such as nucleolocalization peptides or proteins can be used which contain nucleolocalization sequences (Deanwall, et al., TIBS 16:478 (1991)). The utilization of reconstituted viralenvelopes with the above-mentioned nucleoproteins increases the transfection efficiency to a great extent. For example, when an oligonucleotide is mixed with a nucleoprotein it exhibits a further increase in transfection effciency over oligonucleotides that were mixed with a protein such as albumin (Kaneda, et al., Science 243:375 (1989)). Polynucleotides that are operatively linked to a vector such as a plasmid can be more radonmly incorporated into the nucleus of the cells when proteins containing the nuclearlocalization sequences (NLS) pro-lys-lys-lys-arg-lys-val are associated with the vector (Silver, PA Cell 64:489 (1991)). NLSs of 14 amino acids have been attached to micromolecules and even gold particules, and when introduced to the cytoplasm of the cell, they are rapidly incorporated into the nucleos (Findlay, et al. J. Cell Sci. Supp. 11:225 (1989)). The importance of utilizing NLSs in order to facilitate the arrival of the polynucleotides into the nucleus of the cell is supported by data obtained with microinjection. When plasmids are microinjected directly into the nucleus, a greater than 50% transfection of the cells is obtained (Cappechi, Cell 22:479 (1980)).

The efficiency of transfection may also been increased by condensing a polynucleotide with one of various cationic proteins (Tikchonenko, et al., Gene 63: (1988); Bottger, et al. Biochem. Biophys Acta 950:221 (1988); Wagner, et al., PNAS (USA) 87:3410 (1990);Wagner, et al., PNAS (USA) 88:4255 (1991)). The reason for the increasing transfection efficiency may be due to increased cellular up-take of the polynucleotide and/or to dicreased susceptability of the

polynucleotide to nuclease enzymes.

In order to improve the polynucleotide delivery to the nucleus of a cell the length of time that the above molecules remain attached to the polynucleotide is of utmost importance. The polynucleotide may be associated to a receptor ligand by covalent bonding with the ligand to a polycationic polylisine (Wu, et al. (19__), supra). In another embodiment the ligand may be covalently attached to a polynucleotide intercalator, ethidium homodimer (5,5'-diazadeca-methylene bis (3,8-diamino-6-phenylphenanthridium) dichloride dihydrochloride) (NIelsen, Eur. J. Biochem. 122:283 (1982)).

Another cell-assembling polynucleotide delivery system utilizes one or more of DNA-masking components, cell recognition components, charge-neutralization and membrane-permeabilization components, and subcellular localization components Each component in this system is able to perform its indicated function and also assemble or disassemble with the polynucleotide if required.

The following are some of the agents in accordance with the disclosure of the above-referenced application that may be utilized, and the manner in which they are used.

The polynucleotide may be single-stranded DNA or RNA, or double-stranded DNA or DNA-RNA hybrid. Triple- or quadruple-stranded polynucleotides with therapeutic value are also contemplated to be within the scope of this invention. Examples of double-stranded DNA would include structural genes, genes including operator control and termination regions, and self-replicating systems such as plasmid DNA.

The DNA-masking element of this system is a molecule capable of masking all or part of the polynucleotide, thereby increasing its circulatory half-life by inhibiting any attack by degrading reagents present in the circulation.

Polyethylene glycol (PEG) can be covalently linked with a DNA-associating moiety by conventional methods , and used as a DNA-masking component. Alternatively, the DNA may be masked through association with lipids. In one embodiment, the DNA is encased in standard liposomes as described, for

example, in U.S. Patent No. 4,394,448 to Szoka et al., the relevant portion of the specification of which is hereby incorporated by reference. In another embodiment, the DNA is incubated with a synthetic cationic lipid similar to those described in U.S. Patent No. 4,897,355 to Eppstein et al.

The above-described synthetic cationic lipids effectively mask the DNA when associated therewith. Without attempting to limit the invention in any way, it is believed that the lipids form a monolayer structure that encapsulates the DNA in some fashion.

The cell recognition element is a molecule capable of recognizing a component on the surface of a targeted cell, covalently linked with a DNA-associating moiety by conventional methods . Cell recognition components include: antibodies to cell surface antigens, ligands for cell surface receptors including those involved in receptor-mediated endocytosis, peptide hormones, etc. Specific ligands contemplated by this invention include: carbohydrate ligands such as galactose, mannose, mannosyl 5-phosphate, fucose, sialic groups, N-acetylglucosamine or combinations of these groups as complex carbohydrates such as those found on glycolipids of the blood groups or on various secreted proteins. Other ligands include folate, biotin, various peptides that can interact with cell surface or intracellular receptors such as the chemoattractant peptide N-formyl-met-leu-phe, peptides containing the arg-asp-glycine sequence or cys-ser-gly-arg-glu-asp-val-trp peptides, peptides that contain a cystine residue or that interact with cell surface protein such as the human immunodeficiency virus GP-120, and peptides that interact with CD-4. Other ligands include antibodies or antibody fragments such as described by Hertler and Frankel (Hertler, A., and Frankel, A., J. Clin. Oncol. 7: 1932-1942). The specificity of the antibodies can be directed against a variety of epitopes that can be expressed on cell surfaces including histocompatability macromolecules, autoimmune antigens, viral, parasitic or bacterial proteins. Other protein ligands include hormones such as growth hormone and insulin or protein growth factors such as GM-CSF, G-CSF,

erythropoetin, epidermal growth factor, basic and acidic fibroblast growth factor and the like. Other protein ligands would include various cytokines that work through cell surface receptors such as interleukin 2, interleukin 1, tumor necrosis factor and suitable peptide fragments from such macromolecules.

The membrane-permeabilizing element of this system is a molecule that aids in the passage of a polynucleotide across a membrane. The liposomes and synthetic cationic lipids described above as DNA-masking components also may function as membrane-permeabilization components.

The membrane-permeabilizing components of this invention also include polycations that neutralize the large negative charge on polynucleotides. Polycations of this invention include polylysine, polyarginine, poly (lysine-arginine) and similar polypeptides, and the polyamines and the polycationic dendrimers. (Tomalia, D.A. et al., Agnew. Chem. Int. Ed. Engl. 29:138-175(1990)).

In a different embodiment, the membrane-permeabilizing component of the invention may be an amphipathic cationic peptide. Amphipathic cationic peptides are peptides whose native configuration is such that the peptide is considered to have a cationic face and a neutral, hydrophobic face. In a preferred embodiment, the peptide is a cyclic peptide. Examples of the amphipathic cationic cyclic peptides of this invention are gramicidin S, and tyrocidines. The peptide may also contain some or all of the amino acids in the D configuration as opposed to the naturally occurring L configuration.

In a particularly preferred embodiment, the membrane-permeabilizing element includes, in addition to the amphipathic cationic cyclic peptides, a lipid, or a simple polyamine, or both.

The lipid utilized in the invention is an amphipathic molecule which is capable of liposome formation, and is substantially non-toxic when administered at the necessary concentrations either in native form or as liposomes. Suitable lipids generally have a polar or hydrophilic end, and

a non-polar or hydrophobic end. Suitable lipids include without limitation egg phosphatidylcholine (EPC), phosphatidylethanolamine, dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol), cholesterylphosphorylcholine, 3,6,9-trioxaoctan-1-ol-cholesteryl-3e-ol, dimyristoyl-phosphatidylcholine (DMPC), and other hydroxy-cholesterol or aminocholesterol derivatives (see, e.g., Patel, K.R., et al., Biochim. Biophys. Acta 814:256-64(1985)). The lipid is preferably added in the form of liposomes. The added polyamine is preferably spermine or spermidine.

The membrane permeabilizing elements, i.e., the cyclic peptide and optional phospholipid and polyamine, may be added to the composition simultaneously or consecutively. Preferably, the cyclic peptide is added first, and the phospholipid or polyamine added later. The molar ratio of added cyclic peptide to added polyamine is preferably from about 1:1 to about 1:3. The molar ratio of added cyclic peptide to added phospholipid is preferably from about 1:1 to about 1:20.

The subcellular-localization element of this system is a molecule capable of recognizing a subcellular component in a targeted cell, covalently linked with a DNA-associating moiety by conventional methods as described below. Particular subcellular components include the nucleus, ribosomes, mitochondria, and chloroplasts. In a preferred embodiment of this invention, the subcellular-localization component is a nuclear-localization component. The nuclear-localization components include known peptides of defined amino acid sequences, and longer sequences containing these peptides. One known peptide sequence is the SV40 large T antigen heptapeptide pro-lys-lys-lys-arg-lys-val. Other peptides include the influenza virus nucleoprotein decapeptide ala-ala-phe-glu-asp-leu-arg-val-leu-ser, and the adenovirus E1a protein sequence lys-arg-pro-arg-pro. Other sequences may be discerned from C. Dingwall et al., TIBS 16:478-481(1991).

In another embodiment, the subcellular-localization component is a lysosomal-localization component. A known component for targeting lysosomes is a peptide containing

the sequence lys-phe-glu-arg-gln. In yet another embodiment, the subcellular-localization component is a mitochondrial-localization component. A known component for targeting mitochondria is a peptide containing the sequence met-leu-ser-leu-arg-gln-ser-ile-arg-phe-phe-lys-pro-ala-thr-arg.

The DNA-associating moiety of this system refers to a portion of a functional component that interacts in a non-covalent fashion with nucleic acids. The moiety is covalently linked to the rest of the functional components by conventional means or as described below. DNA-associating moieties are preferably major- and minor-groove binders, DNA intercalators, or general DNA binders. In the case of single-stranded polynucleotides, the DNA-associating moiety may even be the linker strand as described above. In such a case the functional moiety, such as the cell-recognition or subcellular-localization component is covalently linked to the linker strand.

In one preferred embodiment, the DNA-associating moiety is a major- or minor-groove binder. The major- and minor-groove binders are moieties known to associate or "lay in" the major or minor groove of DNA. These binders include distamycin A and Hoechst dye 33258.

In another embodiment, the DNA-associating moiety is a nonspecific DNA binder such as a polycation. Polycations include polylysine, polyarginine, poly (lysine-arginine) and similar polypeptides, and the polyamines and the polycationic dendrimers.

In another preferred embodiment, the DNA-associating moiety is a DNA intercalator. DNA intercalators are planar polycyclic molecules such as ethidium bromide, acridine, mitoxantrone, oxazolopyridocarbazole, ellipticine and N-methyl-2,7-diazapyrenium, and derivatives thereof. Biodegradable linkers such as peptides having the sequence -lys-lys- may also be used in attaching the functional component to the intercalator.

The altering DNA fragment may also be operably linked to a vector to form a hybrid vector prior to delivery into the cell. Hybrid vectors, how to produce them, the type of

vector utilized and their construction are well known to persons skilled in the art and need not be further described herein. Hybrid vectors suitable for the practice of the invention may be constructed to include genes which permit the selection of cells having recombinant genes. The hybrid vectors may also include marker genes which allow the quantitative or qualitative identification of cells with recombinant genes. Other facilitating steps include the formation of DNA complexes with calcium phosphate and/or strontium phosphate. The technology for practicing the above steps is generally known in the art and need not be described herein in further detail.

In a preferred embodiment of the in vivo method of this invention, the second DNA fragment is contacted with a recombinase enzyme prior to being administered to the subject. The conditions, ratio and other variables for the preparation of this composition are known in the art.

Recombinases suitable for use herein are UVXS, Rec A, yeast recombinase, human recombinase, and RAD 51 yeast recombinases including DMC and HPPI, among others. In a preferred mode, the second DNA fragment is single-stranded when contacted with the recombinase enzyme. However, the recombinase may be combined with double-stranded DNA.

Briefly, the preparation of the composition of the invention by adding a recombinase enzyme to the altering or second DNA fragment may be conducted as is known in the art, or as is shown in the examples.

The foregoing recombinase agents are useful to increase the frequency of pairing between the intended altering DNA sequence and the cellular DNA. This enhancement of pairing may be useful where the frequency of recombination is found to be low. The use of a recombinase enzyme may also be beneficial to boost the frequency of recombination when the biological application of this method requires relatively high recombination frequencies.

Also a part of this invention is a method for gene therapy of a disease associated with a DNA fragment in a subject's target cells, the DNA fragment comprising a defective DNA

sequence, and first flanking DNA sequences upstream and downstream from the defective DNA sequence, the method comprising

obtaining a second DNA fragment comprising a functional DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the functional DNA sequence, the functional DNA sequence lacking complete homology to the defective DNA sequence, and each second flanking DNA sequence being substantially homologous to the corresponding first flanking DNA sequence, and comprising one terminus of the second DNA fragment, and at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular conditions;

administering to the subject the second DNA fragment under conditions effective for the second DNA fragment to reach the subject's target cells, and allowing the DNA fragment to enter the subject's cells and the second flanking DNA sequences to locate the first flanking sequences; and

allowing the corresponding flanking DNA sequences to pair and anneal and the DNA fragment and the cellular DNA to undergo homologous recombination under cellular conditions to produce genetically functional target cells comprising the functional DNA sequence and substantially ameliorating the disease's symptoms.

The foregoing method may also be used to correct DNA sequences in non-nuclear DNA present in the cell as mitochondrial DNA, a virus, plasmid or a hybrid vector, among others. It will be apparent to a person skilled in the art that this method also provides a means of correcting DNA sequences which do not express a gene product, including promoting the alteration of regulatory genes, intron sequences, and the substitution of redundant codon sequences.

In one embodiment of the method, the functional DNA sequence comprises the wild type or normal gene sequence. Other possible corrections include the insertion of functional DNA sequences comprising other than normal wild-type DNA, but which will permit an at least quasi-normal function and

will have an at least beneficial therapeutic effect on the subject. Generally, the functional DNA sequence needs only be of at least sufficient size and type of sequence to, after homologous recombination, result in a genetic DNA capable of near normal gene expression. The above method may be practiced for inserting an altering DNA sequence of at least about 1000 base pairs. More preferably, smaller DNAs of at least about 100 to 300 base pairs and longer may also be used. More preferably still, DNAs of 1 to 99 base pairs may be used. Moreover, the alteration introduced by the functional DNA may comprise a deletion, in which case the altered cellular DNA will actually be smaller than the original cellular DNA. In the case that the functional DNA cures a deletion in the endogenous sequence, the resulting altered cellular DNA will be longer that the defective one.

The second flanking DNA sequence may comprise at least about 5 or more base pairs, preferably about 10 or more base pairs, and more preferably about 17 or more and up to about 50, and sometimes about 100 base pairs in length and more. Flanking DNA sequences of this length are generally adequate to achieve annealing. The length of the flanking DNA sequences may be varied depending on factors such as the length of the altering sequence, the location of cellular DNA fragments, and the particular base sequence within the flanking DNA sequences.

Random recombination may occur when flanking DNA sequences anneal to similar sequences in cellular DNA other than the ones surrounding the defective DNA sequence. A person skilled in the art will know that increasingly longer flanking DNA sequences will further minimize annealing of altering DNA fragments to short random homologous regions. To minimize random integration, somewhat longer flanking sequences of at least 8 to 17 base pairs are preferred.

The synthesis of the functional DNA fragment may be undertaken by methods known in the art, such as the isolation and separation of wild-type DNA fragments by cleavage with restriction endonucleases, site-directed mutagenesis, de novo oligonucleotide synthesis, and/or a combination of enzyme

restriction and ligation to produce deletions, additions, and the like.

In the in vivo methods, the altering DNA fragment or composition may be administered orally, topically, dermally, subdermally, intratumorally, intracavitarily, subcutaneously, intravenously, intramuscularly, intranasally, or by respiratory inhalation. The agents added to the DNA to form a composition to deliver the drug are described above, and need not be further described herein.

In a further embodiment of the above method the defective sequence is associated with a disease selected from the group consisting of Fanconi's anemia, cystic fibrosis, retinitis pigmentosa, xeroderma pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, Duchenne's muscular dystrophy and Tay-Sachs', among others. However, the method of this invention may also be used to correct defective DNA seqeunces associated with genetic diseases other than those mentioned above. The method only requires that wild-type or other functional DNA sequences be known or that a wild-type or other functional DNA fragment be available from known DNA libraries. Other DNAs may also be used so as to produce an alteration of cellular DNA and an associated modification of the defective gene function.

In a most preferred embodiment, the method of the invention is applied to the correction of the genetic defect associated with cystic fibrosis (CF) disease. The method may also be applied to cells with other genetic defects for which the wild-type or otherwise normal DNA sequence is known. Also within this invention is the correction of DNA sequences associated with diseases in animals other than humans.

The genetic therapy method of the invention may be conducted with a functional DNA sequence that comprises a wild-type genotype lacking the defective sequence causing the disease. Other corrections include the insertion of functional DNA sequences other than normal wild-type DNA, but which will permit at least quasi normal cell function or at least have beneficial therapeutic consequences for the

patient. This type of gene therapy is envisioned to alleviate the most crippling forms of a disease even though not completely curing it.

In another embodiment, the genetic therapy method disclosed herein may also be applied to a defective DNA fragment that comprises an exon having two termini, the exon comprising the defective DNA sequence and each first flanking DNA sequences being provided with one inner terminus that is located at least about 5 base pairs, and preferably at least about 10 base pairs and more, outside the exon's terminus. In this embodiment of the invention, the flanking DNA sequences are placed outside transcribed sequences or sequences to be altered, thus providing a cushion which places any possible errors of initial enzymatic events of replication in non-transcribed sequences. Should mismatching occur during early chain elongation steps, the altered sequences will most likely be placed in introns, thus diminishing the likelihood of introducing further mutations in the corrected or altered gene. Those skilled in the art will know that where the defective DNA sequence is found in the intron sequences, the second flanking DNA sequences are synthesized such that they will anneal to the endogenoues flanking DNA sequences away from the exon, the nearby exon sequences thus resulting entirely between flanking sequences or entirely outside the defective area.

The second DNA fragment for use herein may be a double-stranded DNA fragment, single-stranded DNA fragment and/or single-stranded complementary DNA fragments. The choice of the above DNA fragments depends on the source of the DNA, the method of amplification of the DNA, and/or the experimental/clinical aim of the user of the method. Where the second DNA fragment is double-stranded, the second DNA fragment may be denatured prior to delivery to the cell. The denaturation may be conducted, as is known in the art, by heating and quenching or other methods known in the art.

Also part of the invention is the genetic therapy method wherein the functional DNA sequence differs from the defective DNA sequence by at least one base pair and the genetic

alteration introduced by the functional DNA sequence is selected from the group consisting of a substitution, a deletion, and an addition. The functional DNA sequence may be designed for having substantial homology to a specific defective DNA sequence but lacking the defect resulting in the malfunctioning of the cell. Its synthesis may be undertaken by methods known in the art, such as the isolation and separation of a wild-type functional DNA segment by cleavage with restriction endonucleases, and site directed mutagenesis, de novo oligonucleotide synthesis and/or a combination of enzyme restriction and ligation to produce deletions or additions, and the like as is known in the art.

As already indicated, the functional DNA sequence may be a wild-type genotype lacking the defective DNA sequence causing the functional defect. Alternatively, the functional DNA sequence may be a DNA sequence other than the wild type but also lacking the defective DNA sequence. Such altering DNA sequence may also provide normal or quasi normal cell function and, upon homologous recombination, serve as a functional DNA sequence.

Also within the invention, the second DNA fragment may be administered as a composition comprising the second DNA fragment as a $Ca^{++}/Sr^{++}/PO_4^{=}$ complex, or enveloped by a lipid layer or complexed with a protein and encapsulated by a lipid layer as described above.

The choice of techniques for facilitating the entry of a functional DNA fragment into a target cell will vary depending on the type of cell to be transfected, the frequency of transfection and recombination required, and the number of cells to be transfected.

For the practice of the in vivo method of genetic therapy for correcting a DNA sequence in a target cell, preferred is the method by which DNA fragments are enveloped by a lipid layer or encapsulated in a lipid and/or nuclear protein layer to facilitate its delivery into the cell. Delivery may also be accomplished by microinjection where the cell type is particularly amenable to these techniques. The DNA fragment may also be operably linked to a vector to form a hybrid

vector prior to delivery into a human patient. Hybrid vectors are well known to persons skilled in the art and their construction need not be further described herein. Hybrid vectors suitable for the practice of the invention may be constructed to include genes which permit the selection of cells having recombinant genes. The hybrid vectors may also include marker genes which allow the quantitative or qualitative identification of cells with recombinant genes. In other facilitating steps complexes of the DNA fragment with $Ca^{++}/Sr^{++}/PO_4^{=}$ are formed.

The technology for practicing the above steps is generally known in the art and need not be described herein in further detail.

Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein for purposes of illustration only and are not intended to be limiting of the invention or any embodiment thereof, unless so specified.

EXAMPLES

Example 1: Culture of Cells

The cells were grown either in serum free MLCH-8e (Gruenert, D.C. et al., In Vitro Cell Dev. Biol 36:411 (1990)) or in Eagle's minimal essential medium (MEM) supplemented with 10% FBS, 4mM glutamine, and per ml, and antibiotic. Tissue culture plates were coated with fibronectin, collagen and bovine serum albumin (FN/V/BSA). The growth medium was replaced with fresh medium every other day and the stock cultures were subcultured on reaching 80-100% confluence by trypsinization at a split ratio of between 1:2 to 1:4.

Tracheal epithelial cells were isolated and cultured as described by Greunert et al. (Gruenert, D.C., et al. (1990), supra); Yamaya, M. et al., Am. J. Physiol: Lung Cell Mol. Physiol. 261:L485-L490(1991)). Non-transformed cells were grown in modified serum-free LHC-9 medium (MLHC-8e) on tissue culture plates that had been precoated with fibronectin, collagen, and bovine serum albumin (FN/V/BSA)

(Gruenert, D.C. et al. (1990), supra; Lechner & LaVeck, J. Tissue Culture Methods 9:43 (1985)).

Example 2: Development of Cell Line ΣCRTE29o-

Pure cultures of epithelial cells were transfected as described by Gruenert et al and Cozens et al (Gruenert, D.C., et al., PNAS (USA) 85:5951-5955 (1988); Cozens, A.L., et al., PNAS (USA) 89:5171-5175 (1992)). The cells were grown in 100-mm precoated tissue culture dishes to 70-80% confluence and transfected with the linearized pSVori- vector, a plasmid containing a replication deficient SV40 genome via the calcium phosphate precipitation method. The transfected cultures were grown in MLHC-8e medium at 37°C under 5% CO_2 in air until cells with altered growth characteristics appeared (Gruenert, D.C., BioTechniques 5:740-749(1987); Gruenert,D.C., et al. supra(1988)). Cell transformants were isolated by trypsinization and all the colonies were pooled and designated ΣCFTE29o-.

To enhance the ability of the cells to survive a crisis that generally occurs at about 15 passages post-transfection, the cells were transferred before the crisis occurred to Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and an antibiotic at passage 5-7 post-transfection. The cells entered crisis at passage 17-18 post-transfection. The ΣCFTE29o- transformants were continuously grown post-crisis in supplemented MEM as described above.

Example 3: Immunocytochemical Staining

The cells were grown on well slides (Lab-Tek) coated with FN/V/BSA to various stages of confluence. After washing, fixing, and drying, the slides were rehydrated and stained for immunofluorescence as described previously (Gruenert, D.C., et al. (1988), supra; Cozens, A.L., et al.(1992), supra).

The primary antibodies used in these studies were L19 monoclonal antibody against the SV40 T-antigen. AE1/AE3 anti-cytokeratin antibody (Boehringer Mannheim, Indianapolis,

IN), and E9 monoclonal antibody against the junctional complex adhesion protein, cell CAM 120/80 (Wheelock et al., Cell Biochem 34:187-202(1987). SV40 T-antigen is expressed only in successfully transformed cells and cytokeratin and cellCAM 120/80 are expressed only in epithelial cells.

Example 4: Fluorescence in Situ Hydridization (FISH)

Chromosomal analysis by FISH was carried out as described previously (Pinkel,D., et al., Proc. Nat. Acad. Sci. (USA) 83:2934-2938 (1986); Waldman,F.M., et al., Cancer Res. 51:3807-3813 (1991)).

Briefly, cells were grown on single chamber well slides (LAB TEK) to confluence, washed twice with PBS, and fixed with acetic acid:ethanol (1:3). Slides were covered with 70 % formamide, 2 X SSC, pH 7.0, the cellular DNA was denatured by heating at 70 °C for 2 min. Regions of the slide were selected for chromsome analysis. Cells were then dehydrated sequentially by dipping the slide in 70 %, 85 %, and 100 % ethanol for 3 min each at room temperature. The slide was air-dried and warmed at 37 °C for application of the hybridization mix.

The hybridization mix is as follows.

7 μl, Master Mix #2.1 (5.5 ml formamide, 1 g dextran sulfate, sodium salt, Pharmacia, 0.5 ml 20 X SSC, pH 7.0, final volume 7 ml),

0.5μl carrier DNA (100 μg/ml),

1μl biotinylated repetitive probe (p7ter specific for chromosome 7 centromere (Waldman, F.M., et al. (1991), supra), and

1.5 μl dd H_2O.

The hybridization mix was denatured by heating to 70 °C for 5 min and applied immediately to the prepared slides. The slides were covered with glass coverslips and edges were sealed with rubber cement. The hybridization reaction was carried out in a humidified chamber overnight at 37 °C.

Following the hybridization, the coverslip was removed and and the cells were washed with changes (3 min each) of washing solution (50% formamide, 2 X SCC, pH 7.0), prewarmed

to 45 °C, in PN buffer (0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 to pH 8.0., 1 % Nonidet P-40) prewarmed to 45 °C for 3 min, in PN buffer at room temperature for 3 min. The slides were then stained with 5 μg/ml FITC-avidin in PNM buffer (25 gm nonfat dry milk powder in 500 ml PN buffer) at room temperature for 20 min under a coverslip.

The cells were then washed with 2 changes of PN buffer for 3 min at room temperature and incubated with biotinylated anti-avidin (5 μg in PNM solution) at room temperature for 20 min under a coverslip. The slides were washed with two changes of PN buffer, 3 min each, at room temperature and the cells stained with FITC-avidin (5 μg/ml in PMN solution) at room temperature for 20 min under a glass coverslip. The slides were again washed 2 times, 3 min each, with PN buffer at room temperature. Propidium iodide (2 μg/ml) was then added and the slides were viewed by fluorescence microscopy.

The number of copies of chromosome 7 per cell was determined by counting fluorescent dots per nucleus.

Example 5: Analysis of Cell Lines by Immunocytochemical Staining

Immunocytochemical staining specific for the SV40 T-antigen was found in the all cell nuclei. All cell lines were reactive with an anti-keratin antibody as shown by cytoplasmic staining. The pattern in these cell lines is more diffuse than in most post-crisis clones. Pre-crisis cells showed characteristic pericellular staining with the E9 monoclonal antibody directed against the cell CAM 120/80 epithelial cell adhesion molecule (20) suggesting the presence of junctional complexes.

Example 6: Isolation and Purification of DNA and RNA

Cellular RNA and DNA was isolated as previously described (Laski, et al., Nucleic Acids Res. 10:4609-26 (1982)), (Sambrook, et al., Molecular Cloning: a Laboratory Manual (Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989)).

Cells were washed twice with cold phosphate buffered saline (PBS) and then trypsinized at room temperature to obtain a single cell suspension. The single cell suspension was added

to an equal volume of medium containing 10% fetal calf serum and centrifuged at 1000g. The fetal calf serum serves to inhibit the trypsin and minimizes rupturing of the plasma membrane.

The pellet was washed twice with ice-cold PBS and the cells were then resuspended in Lysis Buffer I. The Lysis buffer contained

0.65% NP40,

10 mM Tris, pH 7.8,

150 mM NaCl, and

1.5 mM $MgCl_2$.

The suspension was vortexed on and off for 10 min on ice. This suspension was centrifuged and the cytoplasmic supernatant separated from the nuclear pellet. The mRNA-containing supernatant was diluted with an equal volume of urea buffer. The urea buffer contained

7 M urea,

10 mM Tris, pH 7.5,

10 mM ethylenediaminetetraacetic acid (EDTA),

350 mM NaCl, and

1% sodium dodecyl sulfate (SDS).

After one extraction with an equal volume of phenol, the RNA was precipitated with 100% ethanol at -20 °C overnight. The RNA pellet was then resuspended in diethyl pyrocarbonate (DEP)-treated H_2O and stored at -70 °C for further analysis. PolyA$^+$ RNA was isolated with a commercially available kit (Pharmacia, NJ).

The nuclear pellet was resuspended in Lysis Buffer II. The Lysis buffer II contained

0.1% SDS,

100 mM NaCl,

40 mM Tris, pH 7.5, and

20 mM EDTA for DNA extraction.

The DNA lysate was incubated overnight at 50°C in the presence of 100 mg/ml proteinase K. After a gentle extraction with an equal volume of phenol, the DNA was precipitated overnight at -20 °C in 300 mM sodium acetate, pH 4.8, with 2 volumes of 100% ethanol. The DNA pellet was washed one

time each with 70% and 100% ethanol, and slowly resuspended in TE buffer. The DNA concentrations were determined spectrophotometrically and the samples were stored at -20 °C.

Example 7: Determination of ΣCRTE29o- Phenotype by Chloride Flux

The chloride ion transport was measured with radioactive ^{36}Cl efflux (Anderson, et al., Cell 66:1027-36 (1991); Cutting, et al., N. Eng. J. Med. 323:1685(1990); Zeitlin, et al., PNAS (USA), in press (1992); Cozens, et al., PNAS(USA)(1992), supra). The cells were grown to confluence in MEM with 10% FBS and the cultures were then rinsed twice with 2 ml efflux buffer. The efflux vuffer contained

140 mM NaCl,
3.3 mM KH_2PO_4,
0.83 mM K_2HPO_4,
1 mM $CaSO_4$,
1 mM $MgSO_4$,
10 mM HEPES, pH 7.4, and
10 mM glucose).

1 ml. fresh efflux buffer with 2 μCi/ml ^{36}Cl was added to each dish, and then cells were incubated for 2 hrs at 37°C. The dishes were washed by dipping each one into 200 ml of efflux buffer for 8-10 seconds.

Following washing, 1 ml efflux buffer was added, and the cells were again incubated at 37°C. Samples were removed at 1 min intervals and replaced with fresh buffer. At 3 min, efflux buffer was added. The efflux buffer contained

10 μM isoproterenol (Sigma; St. Lous, MO), with
500 μM 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphate (CPT-cAMP) (Boehringer-Mannheim, Indianapolis, IN),
10 μM forskolin (Sigma; St Louis, MO), or
0.5 μM ionomycin (Sigma; St. Louis, MO).

Samples containing agonist were removed and replaced at 1 min intervals. The cells were extracted with 0.1 N HCI (1ml) overnight at 4°C and the ^{36}Cl remaining in the cells

was determined.

The samples were counted in scintillation cocktail and the % efflux/1 min time point calculated as follows (Anderson, et al., Cell 67:775-84 (1991)).

$$\% \text{ efflux/min} = \frac{\text{cpm sample}}{\text{average cpm in cells for that minute}}.$$

The average cpm/1 min time interval is $(cpm_t + cpm_{t-1})/2$, where cpm_t are the cpm for the sample at min (t) and cpm_{t-1} are the cpm for the sample at the previous min. (Cone, R.C., PNAS (USA) 81:6349-53 (1984)).

Example 8: Determination of ΣCRTE29o- Phenotype by Whole-cell Patch Clamp

The cells were observed 2-3 days after plating on FN/V/BSA coated glass coverslips. Whole cell patch clamp measurements were conducted as described previously (Hamill, O.P., et al., Pflugers Arch. 391:85-100(1981); Schweibert, et al., PNAS (USA) (1992), in press) at 23°C using a Warner patch clamp amplifier (Hamden, CT, USA). The average seal resistance was 1.2± 0.1 GΩ. The membrane voltage was clamped to a holding voltage of 0 mV and stepped in 10 mV increments using P clamp 5.51 software (Axon Instruments; Foster City, CA). The currents were stored on a Everex AT computer, filtered at 1-5 kHz and analyzed by P clamp 5.51 software. Current magnitude was measured during the last 20ms of an 140 ms voltage pulse. Whole cell capacitance (Cp) was calculated by exponential fit of the RC decay of the transient capacitance spike as described Schweibert (1992) supra; Frindt et al., Am. J. Physiol. 258:F562-F567 (1990)).

The Cp values (in pF) were as follows.

9HTEo−, 6.5±2.3 (n=9);

ΣCFTE29o−, 15.3±1.5 (n=9); and

2CFSMEo−, 16.5±1.7 (n=6).

The pipette solution contained 140 mM N-methyl-D-glucamine Cl, (NMDG-Cl), 1 mM $MgCl_2$, 5 mM HEPES, and 100nM Ca^{2+} (buffered with 1mM EGTA), pH7.4. The bath solution (unless otherwise

indicated) contained 140 mM NMDG-Cl, 1mM CaCl2;5, mM HEPES, and 60 mM sucrose pH 7.4.and 60mM sucrose, pH 7.4.

These solutions were used to prevent a volume induced chloride current as described previously (Cliff et al. Am. J. Physio. 4956-4960(1990); McCann et al., J. Gen. Physio. 94:1015-1036(1989); Worrell et al., Am. J. Physiol. 256:C1111-C1119(1989)). Because these solutions did not contain K^+or Na^+, the observed whole cell currents are referable primarily to Cl^-. CPT-cAMP and ionomycin were added to the bath solution to assay for cAMP- and Ca-dependent Cl^- currents in the 9HTEo- and the ΣCFTE29o- cells. The Cl^- channel blocker used was 4, 4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, Sigma). P values were calculated using paired Students' t-test, unless otherwise indicated.

Example 9: Preparation of the Wild-type 491 Base Pair DNA and Primers.

The 491 bp fragment was generated using the T6/20 plasmid (Rommens, J.M. et al., Science 245:1059-1065(1989)), (ATCC, NJ). The identity of the plasmid was verified by restriction enzyme mapping and further amplified as previously described (Sambrook (1989), supra). After digestion with Eco RI and Hind III, an 860 bp DNA was separated following electrophoresis in 0.8% Seaplaque agarose gel. The fragment obtained contained exon 10 as well as 5' and 3' intron sequences as verified by the restriction enzyme cleavage (Zielinski J., Genomics 10:214-228(1991)). A 50 ng aliquot of the DNA fragment was amplified by the polymerase chain reaction (PCR) using primers CF1 and CF5. (US Patent No. 4,965,188).

The amplified fragment was analyzed on a 1% agarose gel, and then amplified in bulk in 20 separate PCR amplifications each containing 50 ng. The 491 bp fragments were purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction, and precipitated with 100 % ethanol at -70 °C for 30 min. The DNA was centrifuged in an Eppendorf microcentrifuge (14,000 rpm for 10 min at 40 °C). The pellet was washed once with 70 % ethanol and then with 100 % ethanol. After drying,

the DNA was resuspended in dd H_2O at a concentration of 1 $\mu g/\mu l$.

Primers and probes were prepared on automated DNA Synthesizers (Models 390B and 394, Applied Biosystems, Foster City, California).

The sequences of the primers and probes were selected from the published CFTR gene sequence (Zielinski, J., Genomics 10: 214-228 (1991)). The sequences of the primers are shown in Table 1 below.

Table1: Sequences of Primers and Oligonucleotides

CF 1	(S)	5'-GCAGAGTACCTGAAACAGGA-3'	(Seq. ID No. 1)
CF 5	(A)	5'-CATTCACAGTAGCTTACCCA-3'	(Seq. ID No. 2)
CF 6	(A)	5'-CCACATATCACTATATGCATGC-3'	(Seq. ID No. 3)
CF 7	(S)	5'-AGAAAATATCATCTTTGG-3'	(Seq. ID No. 4)
CF 8	(S)	5'-AGAAAATATCATTGGTGT-3'	(Seq. ID No. 5)
CF 9	(S)	5'-ACTTTAAAGCTGTCAAGCCGTG-3'	(Seq. ID No. 6)
CF 17	(S)	5'-GAGGGATTTGGGGAATTATTTG-3'	(Seq. ID No. 7)
CF 22	(A)	5'-CTTGCTAAAGAAATTCTTGCTC-3'	(Seq. ID No. 8)
Oligo N	(A)	5'-CACCAAAGATGATATTTTC-3'	(Seq. ID No. 9)
Oligo ΔF	(A)	5'-AACACCAATGATATTTTCTT-3'	(Seq. ID No. 10)
C16B	(S)	5'-CTTTTCCTGGATTATGCCTGGCAC-3'	(Seq. ID No.11)
C16D	(A)	5'-GTTGGCATGCTTTGATGACGCTTC-3'	(Seq. ID No.12)

Example 10: PCR Conditions

The PCR conditions for individual primers are as follows

CF1/CF5 (491 bp fragment)

10 ng DNA, 10-30 pmole primer, 1.5 mM Mg^{+2}, Cycle: denaturation 94 °C for 1 min, annealing 53 °C for 30 sec, extension 72 °C for 30 sec with a 4 sec increase in the extension time per cycle for 40 cycles.

CF1/CF6 primers (684/687 bp fragment)

0.5 μM primers, DNA 1-2 μg, 1.5 mM Mg^{+2}, Cycle: denaturation, 94 °C for 1 min, annealing, 53 °C for 45 sec; extension, 72 °C for 90 sec with a 4 sec/cycle increase in extension time for 40 cycles.

CF6/CF7/8 (404/407 bp fragment)

1:500 dilution of DNA from CF1/CF6 amplification, 1μM primers, 0.8 mM Mg^{+2}, Cycle: denaturation 94 °C for 30 sec, annealing 49 °C for 20 sec extension 72 °C for 20 sec with a 4 sec/cycle increase in extension time for 35 cycles.

CF17/22 (474 bp fragment CFTR gene 1338-1811),
CF9/CF14 (627bp fragment, CFTR gene 622-1248)

DNA amplified from 1μg RNA (35 cycles), 1 μM primers, 0.8 mM Mg^{+2}, Cycle: denaturation 94°C for 30 sec, annealing 55°C for 30 sec, extension 72 °C for 20 sec increasing 3 sec/cycle for 40 cycles.

CF17/oligo N/oligo ΔF

DNA amplified from 1μg RNA (35 cycles), 1 μM primer, 1.5 mM Mg^{+2}, Cycle: denaturation 94 °C for 1 min, annealing 51 °C for 1 min extension 72 °C for 20 sec with a 4 sec/cycle increase in extension time for 40 cycles.

C16B/C16D

2 to 5 μg DNA, 1 μM primer, 1.5 mM Mg^{+2} cycle: denaturation for 60 s at 94°C, annealing for 45 s at 62°C, and extension at 72°C, starting with 120 sec and increasing to 7 min over 28 cycles.

Example 11: Hybridization of Probes

The hybridization was carried out according to Cozens et al. (Cozens et al(1992), supra). Fragments were separated by agarose gel electrophoresis. The gels with the fragments were incubated in 0.4 N NaOH, 0.6 M NaCl for 30 min to denature DNA and then washed one time with 1.5 M NaCl, 0.5 M Tris-HCl for 30 min.

The DNA was transferred to a Gene Screen Plus membrane (NEN-Dupont) by capillary blot and again denatured with 0.4 N NaOH for 1 min followed by neutralization with 0.2 M Tris-HCl. The membranes were prehybridized for 1 heat 37 °C in 6 X SSC, 5 X Denhardt's, 1% SDS, and 100 μg/ml of denatured salmon sperm DNA.

The oligonucleotide probes (oligo N or oligo ΔF; 10 ng) were radiolabeled by reaction with 20 units of T4 kinase and 40 μCi ^{32}P-γ-ATP for 30 min at 37 °C. Unincorporated nucleotides were removed by centrifugation of the reaction mix through a minispin column.

Hybridization was carried out overnight at 37 °C, the membranes were washed 2 times for 5 min each in 2 x SSC at room temperature, 2 times for 30 min in 2 x SSC, 0.1 % SDS at 45 °C, and 1 time in 0.1 x SSC for 30 min at room temperature. Autoradiographic emulsion was applied to the membranes after washing.

Example 12: Preparation of Denatured DNA for Transfection and Homologous Recombination

The 491 bp fragment was prepared as described, denatured by heating to 95°C for 10 min and then rapidly cooled on ice. 5 μl of the DNA were then added to a buffer solution. The buffer contained

20 mM Tris acetate,

10 mM Mg acetate,

70 mM potasium acetate,

1 mM dithiothreitol, and

100 μg/ml bovine serum albumin-fraction V, 0.5 mM ATP-γ-S)

followed by the addition of

UvsX: 20 μl of a 1.4 mg/ml solution (Formosa, T., et al., J. Biol. Chem. 261:6107-6118 (1985));

UvsY: 36 μl of a 0.5 mg/ml solution (Morrical, et al., J. Biol. Chem. 268:15096-15103(1990)); and

T4 Gen p32: 100 μl of a 5.2 mg/ml solution (Kodadek, T., et al., J. Biol. Chem. 263:9427-9436(1987)).

to a final volume of 250 μl.

The ratio of UvsX molecules to DNA bases to UvsY molecules to T4Gp32 molecules was 4:64:1:17. The fragments were then

coated with UvsX by incubating the solution for 10 min at 37°C and then kept on ice for 10 min before transfection.

Alternatively, the denatured DNA was then added to a 63 μl solution containing

200μγ Rec A protein,

4.8 mM ATP γ-S,

2 mM MgAc, and

1.7 μl reaction buffer (100 mM Tris-Ac, pH 7.5 at 37 °C,

10 mM dithiothreitol, and

500 mM Na/Ac

and incubated for 10 min at 37°C. After 10 minutes, 7 μl of 200 mM MgAc were added to the solution and the fragment was coated with Rec A enzyme at a ratio of 3 bases per Rec A molecule.

Example 13: Identification of ΔF508 Deletion in Genomic DNA

The presence of a ΔF508 mutation in the cellular DNA was tested as described by Kerem, et al. (Kerem et al., Science 245:1073-1080(1989)). The presence of the ΔF508 deletion was determined by preparing genomic DNA by PCR. Using Southern blot hybridization, the PCR amplified DNA was exposed to probes with normal or ΔF508 specific radiolabeled oligonucleotides.

DNA from CF cells known to be heterozygous for the ΔF508 mutation (2CFSMEo-) and from normal cells (16HBE14o-) were used as controls.

Genomic DNA was prepared from transformed epithelial cell lines and amplified by PCR using the GeneAmp kit (Perkin Elmer Cetus, Emeryville, CA). Oligonucleotide primers C16B and C16D (Riordan, et al., Science 245:1066:73 (1989)) were used for amplification of the CFTR region around the ΔF508 deletion site (Exon 10).

The PCR products were separated on 1.4% agarose gels and transferred to Gene Screen Plus membranes (NEN Dupont, Wilmington, DE). Oligonucleotide probes (10 ng each) for the normal CFTR DNA (5'-CACCAAAGATGATATTTTC-3') (Seq. ID No. 9) and for the ΔF508 deletion (5'-AACACCAATGATATTTTCTT-3')

(Seq. ID No. 10) were labeled with γ-[^{32}P]ATP. The filters were hybridized overnight at 37°C with a labeled oligonucleotide probe in a 6xSSC solution (1xSSC contains 150 mM NaCl, 15 mM trisodium citrate, 1% SDS, 1 g/l ficoll, 1 g/l bovine serum albumin fraction V, 1 g/l polyvinylpyrrolidone^{6000}, and 100 mg/ml sonicated salmon sperm DNA. The membranes were washed twice with 2xSSC at room temperature for 5 min, twice at 45°C in 2xSSC, 0.1% SDS for 30 min, and once at room temperature in 0.1xSSC for 30 min. Bands hybridizing to radioactive probes were identified autoradiographicly.

Example 14: Identification of ΔF508 Deletion in Genomic DNA

CFTR exon 10 DNA's from 16HBE14o- (normal), 2CFSMEo- (ΔF508 compound heterozygote CF cell line) and ΣCFTE29o- cells were amplified by PCR. After electrophoresis the DNA fragments were transferred to filters and hybridized with radiolabeled allele-specific oligonucleotide probes. A band from 16HBE14o- cells hybridized with a probe specific for normal 508-510 CFTR sequences. A band from the ΣCFTE29o- cells hybridized with the probe that contains the ΔF508 mutation.

The DNA from the 2CFSMEo- cell hybridized with both probes as this line contained a ΔF508 sequence in one allele and a normal exon 10 sequence in one allele.

Example 15: Determination of CFTR mRNA Expression

Cytoplasmic RNA from 10^6 to 10^8 cells was isolated as described above and the concentration was determined spectrophotometrically. Total cytoplasmic RNA (1μg) was denatured by heating to 95°C for 2 min, and reverse transcribed using the RT-PCR GeneAmp kit (Perkin Elmer Cetus, Emeryville, CA). The first-strand cDNA was PCR amplified from exon 9 to 11 (fragent A) or from exon 5 to 7 (fragment B) (Cozens, PNAS (USA) 89:5171-5175(1992)), using oligonucleotides that span intron-exon boundaries. This eliminates amplification of genomic DNA.

The amplification of fragment A using primers CF17 (sense) and Cf22 (antisense) yielded a 474 bp product (nucleotide positions 1338-1811 of the CFTR gene).

The amplification of fragment B with primers CF9 (sense) and CF14 (antisense) yielded a predicted 627 bp product (nucleotide positions 622-1248 of the CFTR gene) (Cozens et al., PNAS (USA) 89:5171-5175(1992)). The PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide.

Example 16: Demonstration of CFTR mRNA Expression

The expression of the CFTR mRNA in the ΣCFTE29o- cell line was assessed using PCR to amplify cDNA with CFTR specific primers. The presence of CFTR mRNA was confirmed by the presence on gels of the predicted 471 bp band spanning exons 9-11 and a 627 bp band spanning exons 5-7 of the CFTR gene. The level of CFTR mRNA, while not as high as that detected in a highly differentiated bronchial epithelial cell line from a normal individual (Cozens et al., Am. J. Respir. Cell and Mol. Biol., confidential information), is comparable to that seen in other CF and normal airway epithelial cells in that it is readily detectable by PCR (Cozens et al.(1992), supra).

Example 17: Chloride Flux Measurements in Cell Lines

Whole cell patch clamp recordings were made from ΣCFTE29o- cells to characterize the cAMP- and Ca-dependent Cl^- currents. The responses of ΣCFTE29o- cells to cAMP and calcium were compared to a normal control cell line (9HTEo-) and to another CF cell line (2CFSMEo-).

CPT-cAMP (100 μM) was added to the bath solution and failed to increase Cl^- conductance in the ΣCFTE29o- cells, 13.6±1.2 nS (control), and 12.0±1.0 nS (CPT-cAMP); n=9.

CPT-cAMP increased whole cell Cl^- conductance in 9HTEo- cells 11.7±0.9 nS (control), to 20.0±2.8 nS (CPT-cAMP); n=8; $P < 0.005$.

When ionomycin (1μM) was added to medium, it activated outwardly rectifying whole cell Cl^- currents in ΣCFTE29o-

cells. Ca-induced activation was reduced in ΣCFTE29o- cells, as compared to 9HTEo- cells. When analyzed at ±50mV, 9HTEo- cells, the basal slope conductance (G_{Cl}) values were 10.5± 1.1 nS (-50 mV) and 11.4 ±1.3 nS (+50 mV). Ionomycin increased G_{Cl} to 24.0 ± 3.1 nS (-50mV) in normal cells. In ΣCFTE29o- cells, basal G_{Cl} values were 13.3 ±1.6 nS (-50 mV) and 13.8 ±1.6 nS (+50 mV).

Ionomycin-activated Cl^- currents exhibited outward rectification and were inhibited by DIDS (100μM) in 9HTEo- and ΣCFTE29o- cells.

These results were confimed when ΣCFTE29o- and 2CFSMEo- cells were compared to 9HTEo- cells at ±50 mV and showed that an increase in intracellular calcium increases whole cell Cl^- currents in both cell lines. Calcium-dependent regulation of Cl^- in ΣCFTE29o- cells active. However, Ca-induced activation is approximately 40% that of 9HTEo- cells (+ 50 mV). (Figure not shown).

The measurement of Cl^- secretion by efflux of ^{36}Cl confirmed the observations of the patch clamp analysis. The ΣCFTE29o- cells failed to increase secretion of Cl^- in response to isoproterenol with or without CPT-cAMP. In addition, the cells also showed no increase in the rate of ^{36}Cl efflux after stimulation with forskolin. After incubation with the calcium ionophore ionomycin, the ΣCFTE29o- cells showed a significant increase in the rate of efflux, 3-fold greater than that observed in control cells or those exposed to cAMP agonists. It was, however, not possible to determine quantitative differences in the level of Ca-dependent efflux due to individual variation that can not be differentiated using the efflux technique.

Example 18: Chromosome Analysis

The number of chromosomes 7 in existence within transformed cells was carried out to verify the genotype of the cell line and to determine whether gene dosage may play a role in the phenotypic properties of the cells. Chromosome 7 was labeled by in situ hybridization using a chromosome 7 specific DNA probe.

The ΣCFTE29o-. cells showed the presence of 1 (11%) or 2 (70%) copies of chromosome 7 in 81% of the cells.

Example 19: Formation of DNA-Lipid-Protein Complex and Cell Transfection

A DNA protein complex encapsulated by lipid was prepared as described in Legendre (Legendre, PNAS (USA) (1992), in press).

Dioleoylphosphatidylethanolamine (PtdEtn) was used for the preparation of the liposomes. The preparation of liposomes involved drying 4 μM of lipids under nitrogen at room temperature. The lipid film was rehydrated with 4ml of 30 mM Tris HCI buffer, pH 9, and then sonicated for 15 min under argon.

The DNA-protein complex was prepared by diluting 20 μg of DNA (UvsX or rec A coated or uncoated) in 30 mM Tris HCI pH 9 from 20 mg/ml solution in dimethyl sulfoxide. The gramicidin S(40 μg) was added to the DNA and rapidly mixed and then slowly and gently mixed with 175 μl (175 nmoles) of the above lipids.

Cells at 70-80% confluence were incubated in the presence of the lipid-gramicidine S-DNA complex (5 μg per dish) in serum-free MEM for 5 hrs at 37 °C under oxygen with 5% CO_2. The transfection/ incubation medium was then replaced with growth medium (MEM supplemented with 10% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin). The cells were then grown until harvest with daily replacement of medium.

Example 20: Confimation of Occurence of Homologous Recombination with DNA-Lipid- Protein Complex

To determine if cells had undergone homolgous recombination, CFTE29o- cells were grown for 5 to 7 days after transfection with wild-type DNA uncoated and with the same coated by lipid-gramicidin-s. The DNA was isolated and amplified by PCR. The CF1/CF6 oligonucleotide primers were used to amplify the genomic DNA spanning exon 10. This amplification yielded of about 684bp long fragment if the ΔF508 mutation was present in the cells, or about 687 bp long

fragment if the wild-type CFTR sequence was present in the cells.

The amplified fragments were banded by electrophoresis, visualized with ethidium bromide and then transferred to Gene Screen Plus filters (Dupont). The filters were then hybridized with allele-specific normal probes as described previously (Cozens, (1992), supra; Kunzelmann, Amer. J. Resp. Cell and Molec. Biol.(1992), in press).

Southern hybridization of the 684/687 bp DNA fragment was performed with N or ΔF specific probes.

The labeled N oligonucleotide probe hybridized only to sequences that are generated from a normal exon 10. The N probe hybridized to a 684/687 DNA band amplified from the transfected ΣCFTE29o- cells at CFTR exon 10.

This clearly shows that a wild-type sequence had been incorporated into the ΣCFTE29o- cells at CFTR exon 10.

The amplification with the 687 bp fragment was repeated with DNA from normal 56FHTE8o- cells and ΣCFTE29o- cells. The N probe hybridized only to the 56FHTE8o- fragment (containing only N sequences). No cross-hybridization of the N probe to the ΣCFTE29o- DNA, containing only ΔF sequences was seen.

In an alternative method for detecting recombinant sequences, an aliquot of the first PCR amplification was removed for amplification with another set of primers. The primers in this allele-specific amplification were the CF1 and the oligo N or oligo ΔF primers.

The oligonucleotide primers are allele-specific in that each primer sequence spans the region of the ΔF508 mutation. One sense primer contains the wild-type normal (N) sequence at the CFTR 508 location while the other contains the ΔF508 (ΔF) mutation. The CF1/ΔF primer combination produced a 299 bp fragment if the ΔF508 mutation is present in the CFTR DNA. The CF1/N primer combination yielded a 300 bp fragment from the wild-type CFTR DNA.

No 299/300 bp band was detected when the antisense/N combination was used to amplify non-transfected ΣCFTE29o- DNA. However, when the antisense/N combination was used in

transfected ΔCFTE29o- cells a band at 687 bp was detected. This band only occurs if homologous recombination occurs in at least a portion of the transfected ΔCFTE29o- cells, thereby replacing the ΔF508 mutations with the wild-type sequence.

This proves that the CF1/ΔF primer amplified recombinant wild-type sequences in transfected CFTE29o- cells. The amplification with the CF1/ΔF primers gave a 299 bp band with transfected and non-transfected CFTE29o- cells. Some or all of these cells, in addition, ΔF508 mutations. However, the 16HBE 14o- cell DNA amplified with CF1/ΔF primers gave no 299 bp band.

The possibility that the 684/687 bp recombinant band is due to amplification of a residual DNA fragment left in the cell following transfection or to random integration was eliminated by using a primer outside the region of homology. PCR-amplified DNA sequences in the 491 bp region only form a 684/687 bp length DNA if the external primer sequence is integrated adjacent to the 491 bp DNA. This is not the case with randomly integrated 491 bp exon 10 DNA.

Example 21: DNA Transfection by Electroporation and Homologous Recombination

Transfection by electroporation was carried out on CFTE29o- cells using Rec A-coated DNA prepared as described above.

At confluence, the cells were trypsinized and 10^8 cells were suspended in 400 μl of medium in the presence of 5 μg of the Rec A-coated DNA. The cell suspension was kept on ice for 10 min then electroporated at 400 V and 400 μF in a BTX electroporator (BTX Corporation, San Diego, CA). After electroporation, the cells were kept on ice for an additional 10 min and then plated in a T75 flask with an approximate survival of 30 - 50%, and cultured for 5 to 7 days, and then harvested for analysis of DNA and RNA.

Example 22: Results of Transfection by Electroporation and Homologous Recombination

The CFTE29o- cells were grown for 5 to 7 days after transfection with uncoated wild-type DNA and with the same

coated by electroporation. The DNA was isolated and amplified by PCR. The CF1/CF6 oligonucleotide primers were used to amplify the genomic DNA spanning exon 10. This amplification yielded of about 684bp long fragment if the ΔF508 mutation was present in the cells, or about 687 bp long fragment if the wild-type CFTR sequence was present in the cells.

The amplified fragments were banded by electrophoresis, visualized with ethidium bromide and then transferred to Gene Screen Plus filters (Dupont). The filters were then hybridized with allele-specific normal probes as described previously (Cozens, (1992), supra; Kunzelmann, (1992), supra).

Southern hybridization of the 684/687 bp DNA fragment was performed with N or ΔF specific probes.

The labeled N oligonucleotide probe hybridized only to sequences that are generated from a normal exon 10. The N probe hybridized to a 684/687 DNA band amplified from the transfected ΣCFTE29o- cells at CFTR exon 10.

This clearly shows that a wild-type sequence had been incorporated into the ΣCFTE29o- cells at CFTR exon 10.

The amplification with the 687 bp fragment was repeated with DNA from normal 56FHTE8o- cells and ΣCFTE29o- cells. The N probe hybridized only to the 56FHTE8o- fragment (containing only N sequences). No cross-hybridization of the N probe to the ΣCFTE29o- DNA, containing only ΔF sequences was seen.

In an alternative method for detecting recombinant sequences, an aliquot of the first PCR amplification was removed for amplification with another set of primers. The primers in this allele-specific amplification were the CF1 and the oligo N or oligo ΔF primers.

The oligonucleotide primers are allele-specific in that each primer sequence spans the region of the ΔF508 mutation. One sense primer contains the wild-type normal (N) sequence at the CFTR 508 location while the other contains the ΔF508 (ΔF) mutation. The CF1/ΔF primer combination produced a 299 bp fragment if the ΔF508 mutation is present in the CFTR DNA. The CF1/N primer combination yielded a 300 bp fragment from the wild-type CFTR DNA.

No 299/300 bp band was detected when the antisense/N combination was used to amplify non-transfected ΣCFTE29o- DNA. However, when the antisense/N combination was used in transfected ΣCFTE29o- cells a band at 687 bp was detected. This band only occurs if homologous recombination occurs in at least a portion of the transfected ΣCFTE29o- cells, thereby replacing the ΔF508 mutations with the wild-type sequence.

This proves that the CF1/ΔF primer amplified recombinant wild-type sequences in transfected CFTE29o- cells. The amplification with the CF1/ΔF primers gave a 299 bp band with transfected and non-transfected CFTE29o- cells. Some or all of these cells, in addition, ΔF508 mutations. However, the 16HBE 14o- cell DNA amplified with CF1/ΔF primers gave no 299 bp band.

The possibility that the 684/687 bp recombinant band is due to amplification of a residual DNA fragment left in the cell following transfection or to random integration was eliminated by using a primer outside the region of homology. PCR-amplified DNA sequences in the 491 bp region will only form a 684/687 bp length DNA if the external primer sequence is integrated adjacent to the 491 bp DNA. This will not be the case with randomly integrated 491 bp exon 10 DNA.

Example 23: Transfection by Micro-injection and Homologous Recombination

Microinjection was conducted with an Eppendorf 5242 microinjector connected to a Eppendorf 5170 micromanipulator. Borosilicate pipettes (Brunswick, 1.2 mM OD x 1.9 mM ID) were prepared on a Sutter Instruments pipette puller (model p-87). Cells were viewed with an Olympus IMT-2 inverted microscope during injection. The cells were washed 2 times in phosphate buffered saline (PBS) and injected under non-sterile conditions at room temperature in growth medium. The area of injected cells was marked on the side by 2-5 mm diameter rings. The cells were injected with 1000-10000 Rec A-fragments per cell by intranuclear injection with 120 hPa for 0.1-0.3 sec at a volume of 1-10 fl of DNA /nucleus. The cells remained outside the incubator for a maximum of 15 min. After injection, non-injected cells were removed by scraping.

The injected cells were grown for 7 days and then harvested for DNA analysis.

Example 24: Results of Transfection by Microinjection and Homologous Recombination

CFTE29o- cells were grown for 5 to 7 days after transfection by microinjection with Rec A-coated or uncoated wild-type DNA. The DNA was isolated and amplified by PCR. The CF1/CF6 oligonucleotide primers were used to amplify genomic DNA spanning exon 10. This amplification yielded a 684bp long fragment if the ΔF508 mutation was present in the cells or a 687 bp long fragment if the wild-type CFTR sequence was present.

The amplified fragments were banded by electrophoresis, visualized with ethidium bromid and then transferred to Gene Screen Plus filters (Dupont). The filters were hybridized with allele-specific normal probes as described previously (Cozens(1992), supra ; Kunzelmann (1992), supra).

Southern hybridization of the 684/687 bp DNA fragment was performed with N or ΔF specific probes. The labeled N oligonucleotide probe only hybridized to sequences that are generated from a normal exon 10. The N probe hybridized to a 684/687 DNA band amplified from the transfected ΣCFTE29o- cells at CFTR exon 10.

These finding demonstrate that a wild-type sequence was incorporated into ΣCFTE29o- cells at CFTR exon 10.

The amplification with the 687 bp fragment was repeated with DNA from normal cells (56FHTE8o-) and ΣCFTE29o- cells. The N probe hybridized only to the 56FHTE8o- fragment (containing only N sequences). No cross-hybridization of the N probe to the ΣCFTE29o- DNA (containing only ΔF sequences) was seen.

Cells transfected by microinjection permit a minimum estimate of recombination frequency. The presence of at least one recombinant wild-type exon 10 sequence in 4000 microinjected CFTE29o- cells indicates a homologous replacement frequency of $\geq 2.5 \times 10^{-4}$.

In an alternative method for detecting recombinant sequences, an aliquot of the first amplification was then

removed for amplification with another set of primers. The primers in this allele-specific amplification were the CF1 and the oligo N or oligo ΔF primers. The oligonucleotide primers are allele-specific in that each primer sequence spans the region of the ΔF508 mutation. One sense primer contains the wild-type normal (N) sequence at the CFTR 508 location while the other contains the ΔF508 (ΔF) mutation. The CF1/ΔF primer combination produced a 299 bp fragment if the ΔF508 mutation is present in the CFTR DNA. The CF1/N primer combination yielded a 300 bp fragment from a wild-type CFTR DNA.

No 299/300 bp band was detected when the antisense/N combination was used to amplify non-transfected ΣCFTE29o- DNA. However, when the antisense/N combination was used in transfected ΣCFTE29o- cells a band at 687 bp was detected. This band only occurs if homologous recombination occurs in at least a portion of the transfected ΣCFTE29o- cells, thereby replacing ΔF508 mutations with the wild-type sequence. Thus the CF1/ΔF primer amplified recombinant wild-type sequences in transfected CFTE29o- cells. Amplification with the CF1/ΔF primers gave a 299 bp band with DNA from transfected and non-transfected CFTE29o- cells. Some or all of these cells carried ΔF508 mutations. However, 16HBE 14o- cell DNA amplified with CF1/ΔF primers gave no 299 bp band.

The possibility that the 684/687 bp recombinant band is due to amplification of a residual DNA fragment left in the cell following transfection or to random integration was eliminated by using a primer outside the region of homology. PCR-amplified DNA sequences in the 491 bp region will only form a 684/687 bp length DNA if the external primer sequence is integrated adjacent to the 491 bp DNA. This will not be the case with randomly integrated 491 bp exon 10 DNA.

In addition, the possibility that the residual 491 bp fragment remaining in the cell after 6 days and might act as a primer for the PCR reaction was tested. Genomic DNA (ΣCFTE29o-) with the same number of wild-type fragments used during microinjection was PCR amplified. No amplification

to produce a 687bp DNA with the N primer was detected under these conditions.

Example 25: Phenotyp of Recombinants Obtained by Electroporation

Cells transfected by electroporation with Rec A coated 491 bp wild type DNA fragments showed an increase in ^{36}Cl efflux following addition of isoproterenol (10^{-5} M) and CPT-cAMP (5×10^{-4}M).

Nontransfected ΣCFTE29o- cells failed to respond to stimulation with isoproterenol or CPT-cAMP.

Ionomycin (5×10^{-4}), stimulated increased Cl^- efflux in both transfected and non-transfected cells. The increase in efflux, however, was greater in the transfected cells than in the non-transfected cells.

This increased response to ionomycin (also isoproterenol and CPT-cAMP) indicates that transfection of the wild-type CFTR sequence has occurred. These results show a high homologous recombination efficiency.

Example 26: Recombinant DNA Formation from cDNA and Cellular RNA

RNA was isolated from the cells transfected with Rec A-coated DNA using lipid-gramicidins-DNA encapsulation or electroporation and amplified as first strand cDNA. The RNA was denatured by heating at 95°C for 2 min, and reverse transcribed with the PCR RNA Gene Amp kit, according to manufacturer's instructions (Perkin-Elmer/Cetus). First strand cDNA was amplified using primer CF17 at the 5' end of exon 9 and the allele specific oligo N or oligo ΔF. The expected fragments are 322 bp long (CF17/oligo N) and 321 bp long (CF17/oligo ΔF).

The fragments were electrophoresed in 1% agarose gels, ethidium bromide stained and visualized thereafter.

Amplified cDNA generated from control normal (16HBE14o-) and transfected cells yielded 322 bp DNA fragments with the CF 17/oligo N, whereas non-trasfected ΣCFTE29o- cells only showed a 321 bp DNA fragment after amplification with the CF17/oligo ΔF primers, but neither 321 bp nor 322 bp DNA with

the CF17/oligo N primers cDNA from ΣCFTE29o-. Cells transfected by electroporation and amplified with CF17/oligo N primers showed 322 bp bands demonstrating the presence of wild-type CFTR mRNA. In addition, lipid-gramicidin S-DNA transfected cells (with or whitout Rec A coating of the 491 bp DNA) showed the presence of wild-type CFTR mRNA (hybridization of N oligo probe to 322 bp DNA from amplified cDNA).

In addition to the allele specific PCR amplification of first strand cDNA, Southern hybridization analysis was carried out as indicated above. The fragments were transferred to Gene Screen Plus filters and then hybridized with an allele specific (oligo N) probe under the same conditions used for the Southern analysis of the genomic DNA (Cozens et al. (1992), supra; Kunzelmann (1992), supra.

The presence of wild-type CFTR RNA was confirmed autoradiographically in normal (16HBE14o-) control DNA and in DNA of transfected ΣCFTE29o- cells. Southern hybridization of the 322/321 bp cDNA fragments with the ^{32}p-labeled N oligonucleotide probe confirmaed the specificity of the PCR amplification and showed hybridization to amplified DNA from all cells transfected with Rec A coated 491 nucleotide fragment. No hybridization of the N oligonucleotide probe was detected in non-transfected ΣCFTE29o- cells amplified with the CF17/ oligo N or oligo ΔF primers. The N probe hybridized to the amplified cDNA (322 bp) grom 16HBE14o- mRNA. This analysis verifies that the genomic DNA from transfected cells had undergone true homologous recombination at the ΔF508 CFTR locus and expresses wild-type CFTR mRNA.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: COMPOSITION AND METHOD FOR ALTERING DNA SEQUENCES BY HOMOLOGOUS RECOMBINATION

(iii) NUMBER OF SEQUENCES: 12

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(2) INFORMATION FOR SEQ ID No. 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 1:

GCAGAGTACC TGAAACAGGA 20

(2) INFORMATION FOR SEQ ID No. 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 2:

CATTCACAGT AGCTTACCCA 20

(2) INFORMATION FOR SEQ ID No. 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 3:

CCACATATCA CTATATGCAT GC 22

(2) INFORMATION FOR SEQ ID No. 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 4:

AGAAAATATC ATCTTTGG 18

(2) INFORMATION FOR SEQ ID No. 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 5:

AGAAAATATC ATTGGTGT 18

(2) INFORMATION FOR SEQ ID No. 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 6:

ACTTTAAAGC TGTCAAGCCG TG 22

(2) INFORMATION FOR SEQ ID No. 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 7:

GAGGGATTTG GGGAATTATT TG 22

(2) INFORMATION FOR SEQ ID No. 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 8:

CTTGCTAAAG AAATTCTTGC TC 22

(2) INFORMATION FOR SEQ ID No. 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 9:

CACCAAAGAT GATATTTTC 19

(2) INFORMATION FOR SEQ ID No. 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 10:

AACACCAATG ATATTTTCTT 20

(2) INFORMATION FOR SEQ ID No. 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 11:

CTTTTCCTGG ATTATGCCTG GCAC 24

(2) INFORMATION FOR SEQ ID No. 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
86

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 12:

GTTGGCATGC TTTGATGACG CTTC 24

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth herein.

WHAT IS CLAIMED AS NOVEL IN UNITED STATES LETTERS PATENT IS:

1. A composition for altering a DNA sequence flanked by first DNA sequences upstream and downstream thereto, comprising

a substantially pure, isolated DNA fragment comprising an altering DNA sequence, two termini, and second flanking DNA sequences located upstream and downstream from the altering DNA sequence, the altering DNA sequence lacking complete homology to a DNA sequence to be altered, and each second flanking DNA sequence being subtantially homologous to the corresponding first flanking DNA sequence, encompassing one terminus of the isolated DNA fragment, and comprising at least a number of nucleotides effective to attain annealing to the corresponding first flanking DNA sequence under cellular metabolic conditions; and

a biologically acceptable carrier.

2. The composition of claim 1, wherein

the DNA fragment to be altered comprises an exon having one terminus at each end, wherein the exon comprises the DNA sequence to be altered; and

each of the first flanking DNA sequences is provided with one inner terminus that is located at least 10 nucleotides outside the corresponding exon's terminus.

3. The composition of claim 1, wherein the second DNA fragment is selected from the group consisting of double-stranded DNA, single-stranded DNA and single-stranded complementary DNA fragments.

4. The composition of claim 1, further comprising a recombinase enzyme.

5. The composition of claim 1, wherein the second DNA fragment is enveloped by a lipid layer, encapsulated by a lipid and protein layer, and/or a DNA fragment-calcium/strontium/phosphate complex.

6. The composition of claim 1, wherein the DNA sequence to be altered comprises a defective DNA sequence encoding the cystic fibrosis transmembrane regulatory protein; and the altering DNA sequence comprises a functional allele of the transmembrane regulatory protein.

7. A method for genetically altering a double-stranded DNA sequence in a mammalian cell, comprising obtaining a mammalian cell comprising a double-stranded DNA fragment that comprises a DNA sequence to be altered and first flanking DNA sequences upstream and downstream thereto; obtaining the composition of claim 1, wherein the isolated DNA fragment comprises a second DNA fragment;

delivering the composition comprising the second DNA fragment into the cell under conditions effective for the second DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking DNA sequences; and

allowing the corresponding flanking DNA sequences to pair and anneal, and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically altered cell comprising the altering DNA sequence.

8. The method of claim 7, wherein

the DNA sequence to be altered is associated with a disease selected from the group consisting of Fanconi's anemia, cystic fibrosis, retinitis pigmentosa, xeroderma pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, Duchenne's muscular dystrophy, and Tay-Sachs disease.

9. The method of claim 7, wherein

the mammalian cell comprises a human cell.

10. The method of claim 7, wherein

the mammalian cell is selected from the group consisting of non-transformed and immortalized cells.

11. A genetically altered mammalian cell line obtained by the method of claim 7.

12. An ex vivo method for genetically altering a DNA sequence in a subject's target cells, comprising

obtaining non-transformed or immortalized cells from a mammalian subject, the cells comprising a DNA fragment that comprises a DNA sequence to be altered and first flanking DNA sequences upstream and downstream from the DNA sequence to be altered;

obtaining the composition of claim 1, wherein the isolated DNA fragment comprises a second DNA fragment;

delivering the composition comprising the second DNA fragment into the cell under conditions effective for the second DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking DNA sequences; and

allowing the corresponding flanking DNA sequences to pair and anneal, and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically altered cell comprising the altering DNA sequence; and

returning the genetically altered non-transformed or immortalized cells to the subject.

13. An ex vivo method for gene therapy of a disease associated with a defective DNA fragment present in a mammalian subject's target cells, the DNA fragment comprising a defective DNA sequence, and first flanking DNA sequences upstream and downstream from the defective DNA sequence, the method comprising

obtaining target cells from a mammalian subject in need of the therapy, the cells comprising a DNA fragment that comprises a defective DNA sequence associated with the disease and first flanking DNA sequences upstream and downstream from the defective DNA sequence;

obtaining the composition of claim 1, wherein the isolated DNA fragment comprises a second physiologically functional DNA fragment, and the DNA sequence to be altered comprises a defective DNA sequence;

delivering the composition comprising the second DNA fragment into the cell under conditions effective for the DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking DNA sequences;

allowing the corresponding flanking DNA sequences to pair and anneal and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically altered cell comprising the altering DNA sequence of the second species; and

returning to the subject the gene therapy treated target cells having the fuctnional DNA sequence and substantially ameliorating the disease.

14. A method of producing a transgenic, non-human, mammalian animal, comprising

obtaining a germ cell or embryo cell of a non-human, mammalian animal of a first species, the cell comprising a DNA fragment that comprises a DNA sequence to be altered and first flanking DNA sequences upstream and downstream thereto;

obtaining the composition of claim 1, wherein the isolated DNA fragment comprises an altering DNA sequence from a mammalian animal of a second species;

delivering the composition comprising the second DNA fragment into the cell under conditions effective for the DNA fragment to enter thereto and for the second flanking DNA sequences to locate the first flanking DNA sequences;

allowing the corresponding flanking DNA sequences to pair and anneal and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce a genetically altered cell comprising the altering DNA sequence of the second species;

replacing the genetically altered germ cell or embryo cell in a non-human, mammalian animal; and

allowing gestation to proceed to term to thereby produce a non-human, mammalian transgenic animal of the first and second species.

15. A transgenic, non-human, mammalian animal produced by the method of claim 14.

16. An in vivo method for altering a DNA fragment present in a subject's target cells, the DNA fragment comprising a DNA sequence to be altered, and first flanking DNA sequences upstream and downstream from the DNA sequence to be altered, and the method comprising

obtaining the composition of claim 1, wherein the isolated DNA sequence comprises a second physiologically functional DNA sequence and the DNA sequence to be altered comprises a defective DNA sequence;

administering to the subject the composition comprising the second DNA fragment under conditions effective for the second DNA fragment to reach the subject's target cells and allowing the DNA fragment to enter the subject's cells and the second flanking DNA sequences to locate the first flanking DNA sequences; and

allowing the corresponding flanking DNA sequences to pair and anneal under cellular conditions, and the second DNA fragment and the cellular DNA fragment to undergo homologous recombination under cellular conditions to produce genetically altered target cells comprising the altering DNA sequence.

17. The method of claim 16, wherein

the DNA sequence to be altered is associated with a disease selected from the group consisting of Fanconi's anemia, cystic fibrosis, retinitis pigmentosa, xeroderma pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, Duchenne's muscular dystrophy, and Tay-Sachs disease.

18. The method of claim 16, wherein

the DNA fragment to be altered comprises an exon having two termini, wherein the exon comprises the DNA sequence to be altered; and

each first flanking DNA sequence is provided with one inner terminus that is located at least 10 base pairs outside the corresponding exon's terminus.

19. A method for gene therapy for a disease associated with a defective DNA fragment in a subject's target cells, the DNA fragment comprising a defective DNA sequence and first flanking DNA sequences upstream and downstream from the defective DNA sequence, the method comprising

obtaining the composition of claim 1, wherein the isolated DNA fragment comprises a second DNA fragment comprising a physiologically functional DNA sequence and the DNA sequence to be altered comprises a defective DNA sequence;

administering to the subject the composition comprising the second DNA fragment under conditions effective for the second DNA fragment to reach the subject's target cells, and allowing the DNA fragment to enter the subject's cells and the second flanking DNA sequences to locate the first flanking DNA sequences; and

allowing the corresponding flanking DNA sequences to pair and anneal, and the DNA fragment and the cellular DNA to undergo homologous recombination under cellular conditions to produce a physiologically functional target cells comprising the functional DNA sequence and substantially ameliorating the disease's symptoms.

20. An immortalized CF human cell line having ATCC Accession No. CRL 11151.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07917

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01N 63/00; A61K 31/00, 37/00; C07H 15/00; C12N 5/00, 15/00
US CL :424/93B; 435/172.3, 240.2; 514/44; 536/23.1; 800/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93B; 435/172.3, 240.2; 514/44; 536/23.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	CELL, Volume 68, issued 10 January 1992, M.A. Rosenfeld et al., "In Vivo Transfer of the Human Cystic Fibrosis Transmembrane Conductance Regulator Gene to the Airway Epithelium", pages 143-155, see entire document.	1-20
X Y	INTERNATIONAL JOURNAL OF CELL CLONING, Volume 8, issued 1990, S.S. Boggs, "Targeted Gene Modification for Gene Therapy of Stem Cells", pages 80-96, see entire document.	1-20
X Y	SCIENCE, Volume 244, issued 16 June 1989, M.R. Capecchi, "Altering the Genome by Homologous Recombination", pages 1288-1292, see entire document.	1-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 04 November 1993	Date of mailing of the international search report 26 NOV 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer BRIAN R. STANTON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07917

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	CELLULAR AND MOLECULAR BIOLOGY, Volume 37, number 2, issued 1991, M. Gareis et al., "Homologous Recombination of Exogenous DNA Fragments with Genomic DNA in Somatic Cells of Mice", pages 191-203, see entire document.	1-20
X Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 88, issued August 1991, B.C. Trapnell et al., "Expression of the cystic fibrosis transmembrane conductance regulator gene in the respiratory tract of normal individuals and individuals with cystic fibrosis", pages 6565-6569, see entire document.	1-20
E,X Y	US, A, 5,240,846 (COLLINS ET AL.) 31 AUGUST 1993, see entire document.	1-20
X Y	HUMAN GENETICS, Volume 87, number 3, issued July 1991, M.A. Vega, "Prospects for homologous recombination in human gene therapy", pages 245-253, see entire article.	1-20
X Y	EUROPEAN JOURNAL OF BIOCHEMISTRY, Volume 208, issued 1992, K. Roemer et al., "Concepts and strategies for human gene therapy", pages 211-225, see entire article.	1-20
X Y	HUMAN GENE THERAPY, Volume 2, issued 1991, F.D. Ledley, "Clinical Considerations in the Design of Protocols for Somatic Gene Therapy", pages 77-83, see entire article.	1-20
X Y	SCIENCE, Volume 226, issued 26 October 1984, W.F. Anderson, "Prospects for Human Gene Therapy", pages 401-409, see entire article.	1-20
X Y	HUMAN GENE THERAPY, Volume 3, number 3, issued June 1992, J.C. Olsen et al., "Correction of the Apical Membrane Chloride Permeability Defect in Polarized Cystic Fibrosis Airway Epithelia Following Retroviral-Mediated Gene Transfer", pages 253-266, see entire article.	1-20

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Medline
Search terms: homolgous (w) recombination; recombinase?; vivo; cystic (w) fibrosis; gene (w) therapy; Gruenert, Dieter?/au; Kunzelmann, K?/au; cystic

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-13, drawn to DNA compositions and the first appearing method of using said compositions for *ex vivo* modification of cells for administration to an individual, classified in Class 536, subclass 23.1, Class 435, subclass 172.3 and Class 424, subclass 93B.

II. Claims 14 and 15, drawn to the second appearing method of producing transgenic animals and animals thereby produced, classified in Class 435, subclass 172.3 and Class 800, subclass 2, respectively.

III. Claims 16-19, drawn to *in vivo* homologous recombination mediated site-directed mutagenesis, classified in Class 514, subclass 44.

IV. Claim 20, drawn to a particular cell line, classified in Class 435, subclass 240.2.

Claims 7-11 will be examined with any of the inventions of Groups II-VI to the extent that they read on the elected invention.

The inventions are distinct, each from the other because of the following reasons:

The inventions of Groups I-III are distinct one from the other because they are directed towards materially different methods of altering cellular compositions and these methods require overlapping but non-coextensive considerations. For example, the method of Group I is directed towards *ex vivo* manipulation of cells followed by administration of said cells to a recipient organism while the invention of Group III is directed towards direct *in vivo* manipulation of cells within an organism. The *ex vivo* approach to gene therapy requires consideration of administration of cellular compositions which are, by themselves, biologically active, whereas the *in vivo* gene therapy approach requires consideration of targeting of DNA compositions to cells within the body, entry of DNA into cells, recombination of DNA within the target cells with endogenous DNA, etc. Thus, the *in vivo* administration of DNA compositions requires a variety of considerations not necessary for the analysis of *ex vivo* cellular therapy. In addition, the analysis of the efficacy of the two different methods are materially different because the utilization of cellular versus nucleic acids as therapeutic agents is distinct. For example, cellular compositions have inherent biochemical properties whereas nucleic acids require integration into a target cell in order to function. The method of Group II is distinct from either of the methods of Groups I or III because methods of making non-human transgenic animals are materially different from those involved in the modification of somatic cells within an animal. For example, the production of transgenic animals requires consideration of modification of cells capable of giving rise to an intact animal which is not required for consideration of somatic cell modifications.

The invention of Group IV is distinct from the invention of Group I because they represent materially different chemical compositions.

The invention of Group IV and the methods of Groups II and III are distinct because the methods may be used to prepare materially different cellular compositions that are entirely unrelated to cells that have modified CFTR genes.

For the reasons elaborated above, the separate inventions are not so linked by any single special technical feature within the meaning of PCT Rule 13.2 as to be considered to constitute a single invention.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07917

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)
Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.